Project no. PL 517617

Project acronym:
LOTASSA
LOTus **A**daptation and **S**ustainability in **S**outh-**A**merica

Project title
Bridging Genomics and Agrosystem Management: Resources for Adaptation and Sustainable Production of forage *Lotus* species in Environmentally-Constrained South-American Soils.

Instrument: Specific Targeted Research Project

Thematic Priority: Specific measures in support of international co-operation (INCO-DEV)

Title of report

FINAL ACTIVITY REPORT (MONTHS 1-42)

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Project coordinator name: Juan Sanjuán
Project coordinator organisation name: CSIC

Revision [draft 2]

www.lotassa.com

LOTASSA will characterize at the phenotypic and genotypic levels existing plant (cultivated and model *Lotus*) genetic resources, and will develop new genotypes with superior abiotic stress tolerance. The model *L. japonicus* will be used to identify molecular markers associated to stress tolerance that will be later tested in forage *Lotus* species of interest. Global genomic and metabolic responses of *Lotus* to abiotic stresses will be determined. The role of certain genes and particular metabolic pathways in the tolerance of *Lotus* to those stresses will be assessed. Biochemical and genetic markers for *Lotus* tolerance to abiotic stresses will be identified and their validity tested in mapping populations of forage *Lotus*. To optimise nitrogen fixation by *Lotus*, LOTASSA will isolate, characterise and select highly-performing, stress-tolerant bacterial strains for each forage *Lotus* spp. of interest in the targeted constrained environments.

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A summary of the work performed and progress during the third reporting period (25-42 months):

Summary

The LOTASSA Project was an initiative for technical and scientific cooperation between European and LatinAmerican research institutions, under the auspices of the VI European FrameWork Program. The contents and the managing represented an excellent opportunity to undertake collaborative research on forage legumes for marginal soils, an issue of great importance for livestock feeding in the Southern Cone. The project was participated by 16 groups belonging to 14 public Institutions in 4 Southern Cone and 4 European countries. The project was coordinated by Dr. Juan Sanjuan (CSIC, Spain), whom together with Ing. Mónica Rebuffo (INIA, Uruguay) carried out the scientific coordination.

This project took advantage of the close relationship among forage *Lotus* species and the model legume *Lotus japonicus*, aiming to develop new genetic and biotechnological resources to assist and speed up breeding and improvement of plant genotypes well adapted to environmentally constrained environments (drought, salinity, acidity and Al toxicity). The issue of nitrogen fixation was simultaneously approached, through the identification and selection of superior rhizobial strains as inoculants in Lotus pastures adapted to abiotic stresses.

Some of the relevant results achieved by LOTASSA were:

- A collection of *Lotus* species, genotypes and interspecific hybrids characterised at the genetic, biochemical and physiological levels for their responses to abiotic stresses, with all information gathered in an accessible database.
- A collection of hundreds of rhizobial strains from Europe and the Southern Cone, identified at the taxonomic level and characterised for their interactions with forage *Lotus* and the environment.
- A large set of genes and other genetic markers in Lotus associated with the adaptative response to abiotic stresses.
- A wide variety of physiological and metabolic responses of *Lotus* spp. under abiotic stresses.

Many of the tools and biological resources developed during LOTASSA will help future obtaining of plant genotypes and cultivars highly productive in marginal soils and environments. Others shall be useful to produce highly efficient inoculants for Lotus in degraded environments.

This publication details the more relevant results of the project, which are presented as separate chapters to facilitate reading and comprehension, together with a list of all LOTASSA participants.

Chapter 1. Collections and databases

1.1. Introduction

The genus *Lotus* has been historically used in the Southern Cone of South America for pasture improvement. LOTASSA had the objective to contribute to the sustainability of pastures in the region through the generation of biotechnological tools and biological products that allow the development of better adapted genotypes of the forage species *Lotus corniculatus* L., *L. tenuis* Waldst. et Kit. (Syn. *L. glaber* Mill.), *L. uliginosus* Schkuhr (Syn. *L. pedunculatus* Cav.) and *L. subbiflorus* Lag., in soils that suffer drought, acidity or salinity. LOTASSA project took advantage of the model species *Lotus japonicus* (Regel) to develop tools to accelerate breeding of cultivated species. In parallel, the project worked in the identification, characterization and selection of rhizobia strains that could guarantee the optimum N fixation in those soils, thereby improving the productivity, quality and sustainability of *Lotus* pastures in marginal soils.

A fundamental element for the strategy to be successful was to achieve results that were comparable and exchangeable between research groups, as an approach to develop useful tools and increase knowledge in different research disciplines. As each research group had its own experimental methods, the Consortium agreed to share biological materials and results obtained to prevent discrepancies in the development and validation of evaluation methodology. In this way, all the partners used the same plant and bacteria materials as well as research methods.

1.2. The LOTASSA plant collection

The formation of the LOTASSA Collection started well before the Project itself, with germplasm provided by Aarhus University and multiplied in INIA Uruguay. The Collection started with 8 entries (*L. japonicus* ecotypes Gifu and MG-20, *L. filicaulis* Durieu, *L. burttii* Borsos, 3 entries of *L. corniculatus* and 1 entry of *L. uliginosus* 2n; Table 1). The uniform sets of seeds produced according to standardized protocols were, when necessary, delivered to the LOTASSA Collection at INIA La Estanzuela, Colonia, Uruguay. Of course, this is laborious and time-consuming work with no immediate research return. However, the awareness of partners that the participation of each component of the Project was needed for the long-term common good ensured that partners agreed to this program.

By the end of the Project the LOTASSA collection increased up to 886 entries of model and cultivated species, including 239 Recombinant Inbred Lines (RILs) of interspecific hybrids, 7 mutants, 10 check varieties and 24 improved populations of cultivated species. The collection has been active throughout all the period of the Project, with a total of 1446 materials exchanged with all 15 labs that carry out research within LOTASSA.

Table 1. Origin and type of germplasm in the LOTASSA Collection

	Model species				Cultivated species		
Collection status	**Parents**	**Hybrid GxLB**	**Hybrid LFxG**	**Mutants**	**Checks**	**Improved germplasm**	**Hybrids**
Original collection	4	0	0	0	4	0	0
LOTASSA collection	4	151	88	7	10	24	602
Samples Exchanged	101	799	246	47	75	8	170

The collection of cultivated species started with a selection of cultivars and local populations that represented the germplasm commonly used in the Southern Cone, to be utilized as checks. The first materials were *L. corniculatus* cv. San Gabriel, Estanzuela Ganador, INIA Draco, diploid *L. uliginosus* LE 627 from INIA Uruguay, and latter on with the inclusion of *L. corniculatus* Sao Gabriel and UFRGS from Brazil, *L. tenuis* cv. Esmeralda from INTECH, Argentina and Lg 3 and Lg 8 from INIA Chile, *L. uliginosus* ecotypes Lu A and Lu1 from INIA Chile, among others. The Consortium developed 24 divergent populations selected for their performance under drought, acidity or salinity that are described in the corresponding chapters.

Requests for material by partners were made in writing and indicating the proposed use. All materials conserved are free and available to members of the network under MTA. For others, the LOTASSA collection will only be available on the basis of MTA. The sole condition laid down was to report in the *Lotus* Database the data gathered on the germplasm received. This allowed the LOTASSA team to improve our knowledge of the material and its adaptability to different environments.

1.3. The *Lotus* Database

The information was made available using a number of different strategies: project meetings, publishing papers and reports, hosting electronic forum and providing *Lotus* germplasm and rhizobia databases on the LOTASSA web site (Figures 1 and 2). In this context, *Lotus* Database has also been playing an active part in disseminating information and promoting information exchange. This database, that is available exclusively for members of the Project at the present time, was developed by PROCISUR in agreement with the documentation unit of genetic resources of INTA Castelar, led by Dr. Julio Tilleria (Argentina).

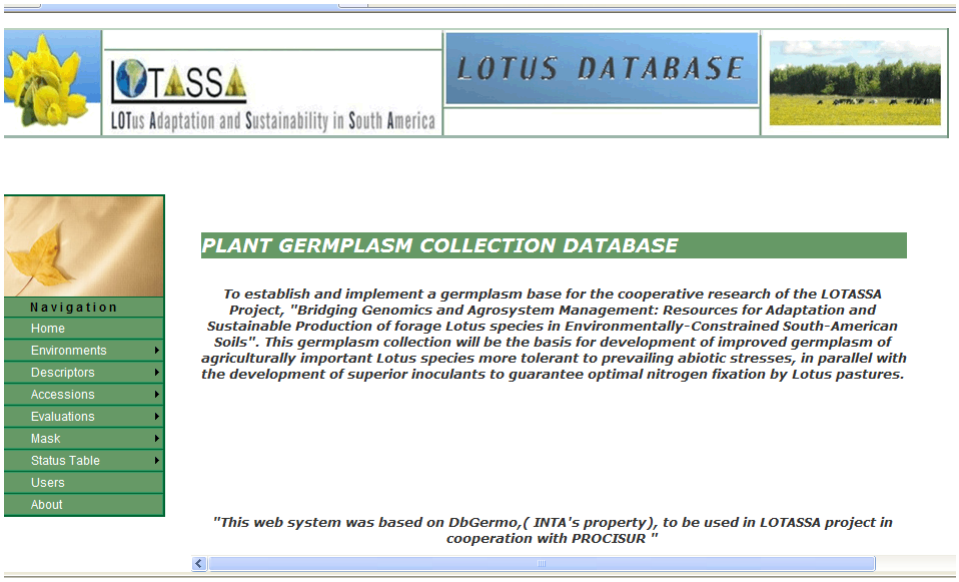

Figure 1. Home page of the *Lotus* Database

Passports databases hold phenotyping information collected from individual and Consortium research groups, rapidly increasing the potential value of the model germplasm and offering the opportunity to exploit the information for breeding of forage legumes. The quantity and quality of information generated by LOTASSA is transferred to its fullest capacity through the development of this relational database with an user–friendly interface that extends beyond simple information storage. The structure of this relational database provides a framework for accessing specific data or a range of information describing a precise germplasm accession. Full utilization of the physiological and genetic information input by the LOTASSA team through the system for other users will provide in the near future the fullest complement of information possible of the *Lotus* Collection, with accurate information for checks and improved accessions characterized for acid, saline and drought stresses.

1.4. Establishment of a *Lotus*-rhizobia collection and associated database

An exhaustive and comprehensive catalogue of microbial resources present in the Southern Cone of South America and other parts of the world was established during the LOTASSA project. That will be the basis for development of improved germplasm of agriculturally important *Lotus* species more tolerant to prevailing abiotic stresses, and to produce superior inoculants to guarantee optimal nitrogen fixation by *Lotus* pastures.

The place for the *Lotus*-rhizobia germplasm collection was established in the Biochemistry Laboratory of the School of Agricultural Sciences, University of the Republic, Uruguay. The laboratory will be in charge of the maintenance and distribution of the rhizobial collection.

A web-based database system for rhizobia collection was developed and the domain http://microorganism.lotassa-databases.org was registered. All the facilities for hosting the operation of the rhizobia database were contracted in order to guarantee the high security levels required as well as the information backup. This application can also be used in other projects with any rhizobia collected from other legume species.

Taking in account that the application could be hosted in web servers using license free platforms, the following technology was used in its development:

Programming language: Java; Database: MySQL. The installation requirements are: Java Platform (version 1.6 or higher); Apache Tomcat WEB Application Server (version 4.1 or higher).

In this database were uploaded the complete data of rhizobia collected during the experimental phase of this project, so far 295 strains and contains all the necessary elements that makes it a useful tool for sharing experimental results among partners of the project and with other scientists in the future.

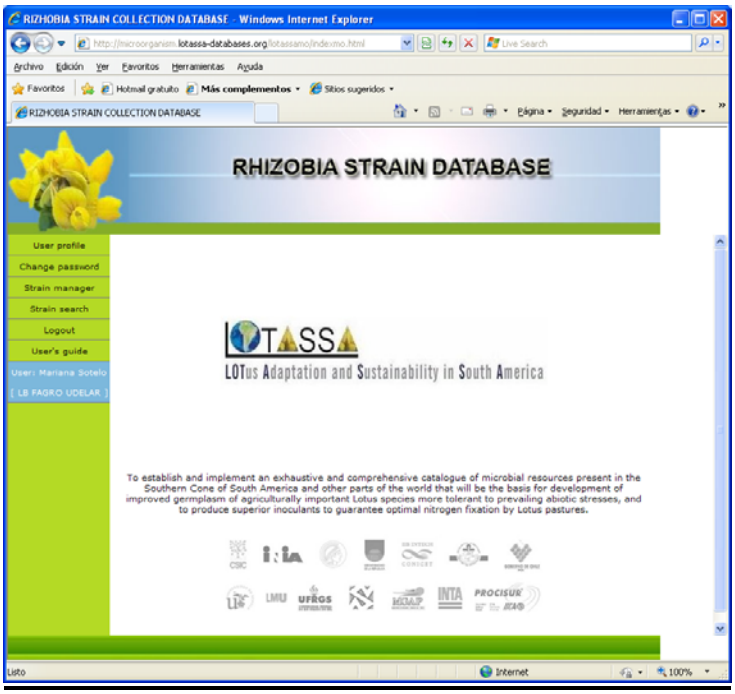

Figure 2. Main page of the Rhizobia Strain Database

Chapter 2. Model *Lotus* species

2.1. Introduction

Legumes play a critical role in natural ecosystems, agriculture, and agro-forestry, where their ability to establish symbiosis with nitrogen fixing rhizobial bacteria makes them efficient colonizers of low-nitrogen environments. Symbiotic nitrogen fixation takes place within nodules on legume roots or stems and app. 90 % of the legume species examined form nitrogen fixing nodules. The symbiotic nitrogen fixation in crop legumes reduces the need for fertilizer and improves soil fertility through crop rotation and intercropping. Moreover, legume seed and foliage in general has higher protein content than non-legumes, and this makes them desirable protein crops. A large part of the nitrogen atoms in living organisms have actually entered the food chain through symbiotic nitrogen fixation.

The first established model plant *Arabidopsis thaliana* (L.) Heynh does not enter into symbiosis with nitrogen fixing microorganisms and phosphorus assimilating mycorrhizal fungi. Therefore two legume species were selected as model legumes: *Lotus japonicus* (*Lj*) which develops determinate nodules like soybean and bean, and *Medicago truncatula* Gaertn (*Mt*) which develops indeterminate nodules like pea and alfalfa. These species have small diploid genomes (around 450 mbp), are self-fertile with ample seed production, can be transformed with *Agrobacterium* and are smaller than most of the agricultural legumes like soybean, pea and alfalfa. Therefore they are easy to use in the laboratory and suitable for molecular genetics. For *Lotus* a substantial fraction of the genome (app. 93 % of the gene space) has been sequenced through BAC and random shotgun sequencing. Moreover the genome sequence of the microsymbiont *Mesorhizobium loti* Jarvis has also been determined http://www.kazusa.or.jp/lotus/index.html

2.2. *Lotus japonicus* ecotypes and related *Lotus* species

L. japonicus ecotype Gifu collected on a river bank in the Gifu prefecture on main-land Japan has been used for many experiments and to create mutant populations from tissue culture and with various types of mutagens.

L. japonicus ecotype MG-20 (Miyakojima) originates from one of the southern islands in Japan. It is used for genome sequencing and for developing the genetic map from *L. japonicus* MG-20 x *L. japonicus* Gifu. Within the ecotypes *L. japonicus* MG-20 has a relatively high level of polymorphisms when compared to the Gifu ecotype. More than 800 microsatellite markers have been developed from TAC or BAC sequences and thereby placing the BACs and TAC in the genome. The microsatellite primer sets have all been developed so that they can be used at the same PCR conditions with annealing temperature of 55 ºC.

In total 91 ecotypes from all regions of Japan can be obtained through LegumeBase http://www.shigen.nig.ac.jp/bean/lotusjaponicus/wildStrainListAction.do They have for example been used to find nematode resistance in the various lines. Grant and coworkers showed that several diploid species of Lotus can be crossed to *L. japonicus* and give fertile offspring. This is another important source of phenotypic and genetic variation.

One of the best crossing partners turned out to be an accession of *L. filicaulis* which originates from Algeria. *L. filicaulis* and *L. japonicus* Gifu show many phenotypic differences and high level of polymorphism on the DNA level (AFLP, SSR). Development of the F2 population and subsequent generations was difficult due to low seed set. The high level of polymorphism in this cross was used in map based cloning of the hypernodulation gene *Har1* and for the Nod factor receptor gene *Nfr5*. Now the number of SSR markers from *L. japonicus* MG-20 x *L. japonicus* Gifu is so high that such a cross is always used in mapping and map based cloning unless the phenotype of MG-20 differs from Gifu for a trait of interest in a way that makes mapping difficult.

The genetic map from *L. filicaulis* x *L. japonicus* Gifu showed the same marker order (colinearity) as for *L. japonicus* MG-20 x *L. japonicus* Gifu but with variations in the cM distances in some of the regions. So both types of crosses and maps help in assigning the marker and sequence order in the genome of *L. japonicus*.

L. burttii originates from Pakistan. It is a good crossing partner for *L. japonicus* as it gives better seed set, shorter generation times and have fewer chromosome regions with distorted segregation than *L. filicaulis* x *L. japonicus* Gifu. *L. krylovii* Schischk appears to be as close to *L. japonicus* as *L. burttii* and closer than *L. filicaulis*. Aarhus University have obtained an F2 population of *L. japonicus* Gifu x *L. krylovii*. This population could also be used for mapping of various phenotypic traits.

The evolutionary relationship between the different *Lotus* species based on nrITS phylogeny has been already determined. As part of the LOTASSA collaboration Aarhus University has further investigated the level of polymorphism by sequencing 17 full length *L. tenuis* cDNA clones isolated by INIA/UACH in Chile. These cDNA clones represent genes that are differentially expressed during drought stress of *L. tenuis* root nodules. This sequence information gives an indication of the level of polymorphism between *L. japonicus* and *L. tenuis*. The sequence identity between the coding regions of these genes in *L. tenuis* and *L. japonicus* is above 95% showing how closely these Lotus species are related. This again emphasizes that the genetic information obtained in the model species *L. japonicus* is of use in other Lotus species such as *L. tenuis*.

2.3. Recombinant inbred lines (RILs) and QTLs

F2 populations of different hybrids were further developed to recombinant inbred lines (generation F8) where the lines are mainly homozygote. Thereby the mapping results can be shared between laboratories and experiments can be repeated on genetically identical material.

For the characterization of quantitative traits (QTLs) Aarhus University have developed two types of RILs. For the *L. filicaulis* x *L. japonicus* Gifu RILs the number of lines in the mapping population that has reached F8 is 116. A total of 175 RILs of *L. japonicus* Gifu x *L. burttii* have reached generation F8. In addition RILs for *L. japonicus* Gifu x *L. japonicus* MG20 have been developed by Kazusa DNA Research Institute and made available through LegumeBase. http://www.shigen.nig.ac.jp/bean/lotusjaponicus/top/top.jsp In this population a number of agricultural traits have been scored and mapped.

L. filicaulis x *L. japonicus* Gifu RILs

The total number of markers that have been used for mapping has been increased to 222 markers, mainly microsatellite markers. The marker scoring was done

in the laboratory of Aarhus University. The mapping data can be obtained from nns@mb.au.dk.

L. japonicus Gifu x *L. burttii* RILs:

A total of 175 RILs of *L. japonicus* Gifu x *L. burttii* have reached generation F8. DNA has been made from 169 of these lines. Microsatellite marker scoring was done in collaboration with Brachmann and Parniske, Ludwig-Maximilians-Universität, Munich, Germany and Sato and Tabata, Kazusa DNA Research Institute, Japan. The total number of scored markers with known map position is now 102 markers evenly distributed over the chromosomes. This density gives a good background for mapping of quantitative trait loci (QTL). The mapping data are available from nns@mb.au.dk. Additional microsatellite markers are continually being scored.

In the future these lines and the *L. filicaulis* x *L. japonicus* Gifu RILs will also be made available to the public through LegumeBase in Japan from where the *L. japonicus* Gifu x *L. japonicus* MG-20 RILs can already be obtained http://www.shigen.nig.ac.jp/bean/lotusjaponicus/top/top.jsp

The *L. japonicus* Gifu x *L. japonicus* MG-20 RILs mapping data are available from the web site of Kazusa DNA Research Institute. These three types of RILs are now used to analyze for example root nodule symbiosis with various strains, drought, pH and NaCl stress responses (LOTASSA) and metal content. Quite high levels of differences are seen among the parents.

The *R. leguminosarum* DZL strain makes a Nod factor that in addition to the normal *R. leguminosarum* Nod factor contains an acetyl fucose moiety which is also found on the Nod factor of the normal symbiont of *L. japonicus, Mesorhizobium loti*. The *R. leguminosarum* DZL strain gives Fix- nodules on *L. japonicus* Gifu but no nodules on *L. filicaulis*. Therefore, it was possible to use the corresponding RILs to map this trait to the lower part of chr. 2 in a region containing the *Nfr5* Nod factor receptor gene. In a more detailed study the University of Aarhus showed that a particular amino acid of a LysM domain of Nfr5 is very important for recognition of the *R. leguminosarum* DZL strain based on the difference in nodulation between *L. japonicus* and *L. filicaulis*.

The growth response during NaCl stress for different *L. filicaulis* x *L. japonicus* Gifu RILs has been determined by Melchiorre et al. IFFIVE-INTA, Argentina. Aarhus University used the program QTL Cartographer to look for QTLs based on the phenotypic (length and relative length) and mapping data. Significant QTLs on chomosome 1, 3 and 5 were detected.

2.4. DNA markers: Synteny

One of the ideas behind the model legume concept was to exploit synteny between model and crops to accelerate isolation and comparative characterisation of genes from the less characterised crop legumes. This approach requires that the target genome regions contain the same genes in approximately the same order in models and crops. In the legume anchor project, Aarhus University have identified a number of single copy genes from legumes based on EST sequences from soybean, *Medicago truncatula* and *L. japonicus*. A criterion for selection of a gene as a legume anchor marker candidate (Leg marker) is also that it is a single copy gene in *Arabidopsis*. Such

genes are ideal for the analysis of homologous/orthologous genes from different legumes to look for the level of synteny.

Using Leg markers, Aarhus University have anchor tagged loci covering 758 cM of the bean (*Phaseolus vulgaris* L.) genetic map and a set of 99 shared loci made it possible to compare this map with the genetic map of *Lotus*. All of the eleven bean linkage groups had non-interrupted regions of at least two markers also showing linkage in the *Lotus* genome, and in several of the bean linkage groups synteny spanned entire linkage groups. In *Pv*LG7 all nine Leg markers with a known position on the genetic linkage map of *Lotus* map to *Lj*LG5. On *Pv*LG11 eleven out of twelve markers map to *Lj*LG3, and on *Pv*LG2 eleven out of twenty markers map to *Lj*LG4, whereas the remaining nine markers map to *Lj*LG2. All in all the legume anchor markers revealed a broad conservation of gene linkage (macrosynteny) between bean and *Lotus*. This is in agreement with previous data based on genome sequences of *L. japonicus*, *M. truncatula* showing ten areas with extensive synteny covering a major part of the total genomes of these species.

In addition, the University of Aarhus used the legume anchor approach to look for synteny to the distantly related legume groundnut (*Arachis hypogeae* L.). In this case, as well the study was able to show synteny between *Arachis* species, *Lotus* and *M. truncatula*, but less pronounced compared to bean as expected.

It is therefore clear that synteny can be used as an analytical tool in both scientific and practical aspects of legume research. The substantial genome information from *Lotus*, *Medicago* and soybean can be used as a help for genetic mapping and gene isolation in other legumes. Recently a comparative mapping approach was used for a complex disease resistance gene locus from bean and the virus resistance gene Rsv4 in soybean. In addition a number of genes for basic traits in addition to symbiotic genes will be easier to isolate from model legumes and afterwards the corresponding gene can be identified and followed in breeding programmes in crop legumes.

2.5. DNA markers for *Lotus* species

Microsatellite markers in LOTASSA *Lotus* species

Microsatellites are mainly made from repeats of di or tri nucleotides. They are mainly placed in noncoding regions and the copy number changes rapidly during evolution. Using standard PCR conditions, 78 of the microsatellite markers developed from *L. japonicus* Gifu x *L. japonicus* MG-20 have been tested on DNA of *L. corniculatus*, *L. tenuis*, *L. uliginosus* and *L. subbiflorus* (Figure 3). In many cases products are amplified from these species. Such molecular markers are potentially useful for breeding with the Lotus species used in agriculture. The map positions in *L. japonicus* are already known for these microsatellite markers. In total several hundreds of microsatellite markers have been developed by Kazusa DNA Research Institute and mapped to a specific position on the Lotus genome.

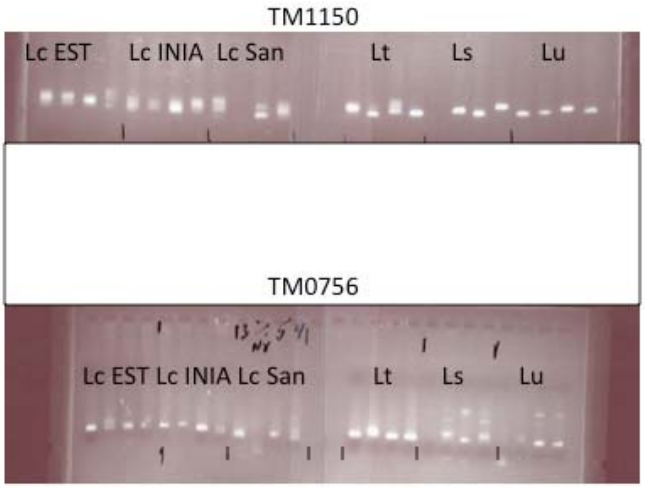

Figure 3. *L. japonicus* microsatellite markers (TM1150 and TM0756) tested in individual plants of *L. corniculatus* (Lc), *L. tenuis* (Lt), *L. subbiflorus* (Ls) and *L. uliginosus* (Lu).

Since there is synteny between *L. japonicus*, common bean and *M. truncatula* and also clearly with the more distantly related legume groundnut, there will probably be very high to the other Lotus species. Therefore, it will expected that markers that are linked in *L. japonicus* will also be linked in most of the other Lotus species.

Conclusion about microsatellite markers:

Most of the *L. japonicus* microsatellite markers amplify well in *L. corniculatus* and *L. tenuis* and several of them amplify well in *L. uliginosus* and *L. subbiflorus*. This analysis confirms that *L. japonicus* is closest to *L. burttii* and *L. filicaulis* followed by *L. corniculatus* and *L. tenuis* and then finally *L. subbiflorus* and *L. uliginosus*.

The level of polymorphism is high between breeding lines and even within breeding lines. Some of these markers could therefore be used in breeding. Furthermore these markers are useful for testing of crosses between different *Lotus* species and lines.

Molecular markers for testing of crosses between *L. uliginosus* and *L. japonicus*:

From the comparison of *L. uliginosus* sequences available at NCBI to *L. japonicus*, a marker was developed giving a product that is 12 bp larger in *L. uliginosus* than in *L. japonicus* As this marker is developed from a sequence that is known from both species it performs better than most markers developed only from the knowledge of the *L. japonicus* sequence such as the microsatellite markers.

2.6. *Lotus japonicus* mutants and genes

Symbiotic mutants with a Nod$^-$ phenotype

Several classes of non-nodulating *L. japonicus* mutants lacking any of the characteristic physiological and cellular responses to rhizobia or arrested in the early signal transduction pathway have been isolated and characterized. Perception of the bacterially produced Nod factor signal is mediated by the Lys motif (LysM)–containing

Nod factor receptors, NFR1 and NFR5, and both receptors are required for the host plant to initiate the physiological and cellular responses leading to infection and nodule organogenesis. The Univ. of Aarhus have shown that the LysM domains of the Nod factor receptors NFR1 and NFR5 are determinants of host specificity through lipochitin-oligosaccharide recognition and furthermore that transfer of the *L. japonicus* receptors to *Medicago truncatula* extended the host range to include *M. loti*. Using a *Rhizobium leguminosarum* DZL strain synthesizing acetyl fucosylated Nod-factor perceived by *L. japonicus* but not *L. filicaulis* it was demonstrate that the LysM2 domain of NFR5 is involved in recognition of the Nod-factor and that a single amino acid substitution can change Nod-factor recognition.

L. japonicus is nodulated efficiently with *Mesorhizobium loti* strains but not by *Bradyrhizobium* sp. NZP2309. In contrast *L. uliginosus* is nodulated efficiently by NZP2309, but not by *M. loti*. In collaboration with University of Copenhagen and Carlsberg Research Center, Denmark, the University of Aarhus has determined the Nod factor structure for *Bradyrhizobium* sp. NZP2309. This Nod factor contains an additional carbamoyl group compared to the Nod factors synthesized by the standard *Mesorhizobium loti* strains. Although the corresponding LysM domains of Nod factor receptors (NFR1 and NFR5) of *L. japonicus* and *L. uliginosus* contain amino acid differences, the results through transgenic plants show that the differences in the Nod factor receptor amino acid sequences are not responsible for the difference in nodulation phenotype of the two rhizobial strains.

2.7. Biotechnological application of abiotic stress responsive genes in *Lotus japonicus*

The University of Munich investigated the applicability of known regulatory genes in the engineering of *Lotus* plants in order to develop genotypes with superior abiotic stress tolerance. A number of key genes, mostly identified in *Arabidopsis thaliana*, are involved in the regulation of stress tolerance. Most of the identified genes are positive regulators of abiotic stress responses. Ectopic expression of these genes in other species or its overexpression can lead to an enhanced abiotic resistance phenotype. In opposition to this, mutations in negative regulators can lead to a more resistant behaviour under abiotic stress conditions. Some genes have been isolated in *A. thaliana* whose mutations benefit the plant under certain situations. DREB1C is a DRE binding transcription factor. It is suggested that DREB1C is a negative regulator of salt, drought and freezing stresses. MYB60, an R2R3 transcription factor that belongs to the MYB superfamily, is involved in stomata movement. *Myb60* knocked-out mutants are more tolerant do desiccation. Meanwhile, CBL1 is a calcium sensor calcineurin B-like protein and a central regulator of abiotic stress responses in *Arabidopsis thaliana*. Based on over-expression and mutant plants analysis, it has been suggested that CBL1 is a positive regulator of salt and drought responses and a negative regulator of cold responses in plants.

Therefore, the Univ. of Munich chose *MYB60*, *DREB1C* and *CBL1* as candidates for biotechnological engineering approaches in *Lotus.* The whole genomic sequence of the putative orthologue genes were obtained in collaboration with Shusei Sato and Satoshi Tabata (Kazusa DNA Research Institute, Japan). The TILLING (Targeting Induced Local Lesion IN Genomes) approach was used to screen an EMS (ethyl methanosulfonate)-mutagenized population of *L. japonicus* for plants that bear mutations in the chosen genes at the John Innes Institute, Norwich, UK. This method

can generate a range of mutant alleles that can provide heterogeneity of phenotypes and insight in gene function.

Nine plants with mutations in *DREB1C* were identified. Four of the plants contain silent mutation whereas the other five carried missense mutations (Table 2). The allelic series is therefore promising a range of phenotypic strength among which the most applicable one can be chosen upon field testing. Three plants with mutations in *MYB60* were obtained; two of them have a missense mutation. The TILLING on *CBL1* identified five lines with mutations. Four of the lines carried silent mutations and a fifth plant contained a missense mutation. The phenotypes under stress conditions are going to be studied on plants homozygous for the mutation. Most of the studies in abiotic stress responses have been done in the model plant *Arabidopsis thaliana*. The results that will come out from our approach may support the importance of targeted genes, previously identified in model plants, in agronomical important species such as *Lotus* sp. with the aim of facilitating breeding and selection of Lotus genotypes more resistant to environmental constrains.

Table 2. *L. japonicus* lines carrying mutations in abiotic stress genes identified through TILLING. *HET; heterozygous, HOM; homozygous*

Gene	Plant name	HET/HOM	Mutation change	Missense	Additional characteristics
dreb1C	1811-1	HET	G/A	D75N	Low seed production
dreb1C	1222-1	HOM	G/A	D121N	
dreb1C	1396-1	HET	C/T	A69V	Very low seed production
dreb1C	1145-1	HET	C/T	A69V	Low seed production and only from HET
dreb1C	1202-1	HET	C/T	L52F	No germination
myb60	728-1	HET	C/T	P283S	Low survival rate
myb60	1158-1	HOM	C/T	P284S	
cbl1	2100-1	HOM	C/T	S140L	

Chapter 3. Investigations on hydric deficit in *Lotus*

3.1. Introduction

The identification of biochemical and physiological characters which contribute to improve the yield in forages legumes under environmental limiting conditions is a main objective of plant breeders for agricultural and cattle rearing regions of South America. Among these forage legumes different *Lotus* species, mainly *L. corniculatus*, *L tenuis* and *L. uliginosus*, are widely used in order to improve the forage yield in the pastures. These species are chosen because of their capacity to adapt to different environments that suffer abiotic stress conditions, such as high salinity, acidity and drought. In particular, drought is one the most frequent problems affecting plant productivity and persistence of *Lotus* species used in forage production.

Improvement of plant breeding programs in *Lotus* can be assisted by the use of other *Lotus* model legume species such as *L. japonicus* ecotypes Gifu and MG-20, *L. filicaulis*, and *L. burttii,* as a result of recent advancement of genomics and functional genomics tools. Information obtained in the model plants can be transferred to the cultivated ones.

We present here a briefing of the results produced in LOTASSA project related to selection of drought tolerant populations of *Lotus* and the analysis of physiological and molecular markers of drought stress.

3.2. Selection of *Lotus* spp. drought tolerant populations in Chile

The narrow-leaf trefoil, *Lotus tenuis*, is naturalized in central Chile mainly in the zones for rice crop on clayed soil. Glasshouse experiments for characterizing *L. tenuis* populations, as adult plants and as young plants at their early developmental stages, under several soil water availability (SWA) levels were carried out by INIA Chile. These experiments compared eleven Chilean populations collected in 1998 and 1999 between the 33° to 38° of south latitude where the species grows spontaneously. In the adult plants, the relative rate of stem elongation (RRSE), the dry matter (DM) production, the relative water content and the specific leaf area showed a significant reduction in the treatment with the highest water restriction (10% SWA). The drought sensitivity index varied broadly among populations, from 0.49 to 1.34 (Figure 4), and was correlated negatively with DM production under water stress (10% SWA). In the young plants the RRSE increased more than 100% when SWA was increased from 10 to 100%. It was concluded that *L. tenuis* population number 4 (collected in San Javier, 35°39’28’’ S) was the most drought tolerant and the number 14 (collected in Melipilla, 33°39’58’’ S) the most sensitive to drought.

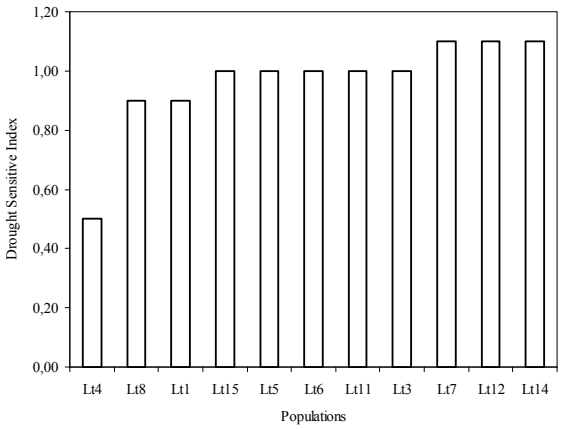

Figure 4. Drought sensitive index of eleven *Lotus tenuis* populations naturalized in Chile.

3.3. Selection of *Lotus* spp. drought tolerant populations in Uruguay

Lotus corniculatus (Lc) cv. San Gabriel and INIA Draco and *Lotus uliginosus* (Lu) cv. LE627 nurseries with more than 1800 plants each, were explored for water stress tolerance and root morphology diversity in greenhouse experiments under water stress (40 % field capacity) by INIA Uruguay. Contrasting plants were selected on yield performance under water stress, integrating sensitive (Lc-C1S) and tolerant (Lc-C1T) populations. 50 plants selected as tolerant produced 92% more forage than 43 plants that integrated the sensitive selection under water stress. Similarly, 56 plants selected for the sensitive (Lu-C1S) population produced less than half compared with the selected tolerant plants (Lu-C1T). Some of these populations are available in the LOTASSA collection.

Root characteristics were also recorded for all plants after the third forage evaluation of the nurseries of *L. corniculatus* and *L. uliginosus* under drought stress. The presence and absence of fibrous lateral roots (>1 mm diameter) up to 4 cm depth were the main parameter of selection in the root system, yielding 42 and 39 plants that integrated the tap-root (LcC1TR) and branch-root (LcC1BR), with an average of 1.14 and 5.82 lateral roots per plant respectively. There were small differences in the number of fibrous lateral roots in Lu, selecting 31 and 46 plants for Lu-C1TR and Lu-C1BR respectively. 34 plants with high density of small roots were selected for cross-pollination as a third Lu population (Lu-C1VR).

4 divergent populations of *L. corniculatus* and *L. uliginosus* for forage production under water stress, as well as 5 populations with different root characteristics were sown in the fields for further tests (Figure 5a). *L. corniculatus*, a tolerant species, was more productive than Lu under the first severe summer drought (harvests Nov-08, Dec-08, Feb-09). These evaluations have shown that the selection for tap root phenotypes provided the most consistent benefits for both species under field drought conditions (Figure 5b). So far, one cycle of massive selection for forage production under water stress has not produced changes in forage production under field conditions. There was moderate to severe drought from the establishment of the experiment onward, conditions where the tap root selection probably explored water available deeper in the soil than other root type systems.

Figure 5a. Large differences in forage production between *L. corniculatus* and *L. uliginosus* selections were recorded in December 2008 at INIA La Estanzuela, Colonia, Uruguay.

Figure 2b. Plots of *L. uliginosus* (top) and *L. corniculatus* (bottom) with the tap root selection (left) compared with check cultivars (right).

3.4. Comparison of drought stress response between cultivated and model species

The cultivated *Lotus* spp. (*L. corniculatus, L. uliginosus* and *L. tenuis*) were compared with the model *Lotus* spp. (*L. japonicus,* Gifu and MG 20, *L. burttii* and *L. filicaulis*) under two levels of SWA. All the measured characters were affected by the water availability and showed significant differences among populations. *L. corniculatus* and *L. tenuis* showed the highest values of DM accumulation (Figure 6) under water stress, therefore, they would be the most drought tolerant species. *L. burttii* and *L. japonicus* ecotype MG-20, were the most drought sensitive species. *L. corniculatus* had the higher leaf stomatal conductance and stem water potentials, which would mean that the species possess osmotic adjustment mechanisms which allow the plant to maintain the transpiration rate with high water tension in its tissues.

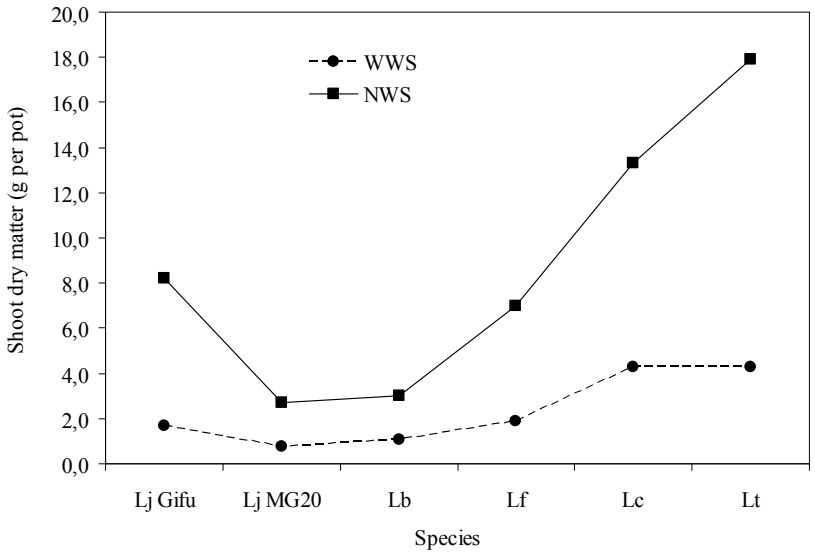

Figure 6. Shoot dry matter production of cultivated and model *Lotus* spp. under two level of soil water availability. With water stress (WWS) and non water stress (NWS). Lj, *Lotus japonicus;* Lb, *Lotus burttii*, Lf, *Lotus filicaulis;* Lc*, Lotus corniculatus*; Lt, *Lotus tenuis.*

In addition, *L. japonicus* Gifu and MG20, *L. filicaulis* and *L. burttii* were phenotypically characterized in the greenhouse for drought tolerance and root morphology, together with *L. corniculatus* cv. San Gabriel, *L. corniculatus* cv. INIA Draco and *L. uliginosus* LE627 under 4 water regimes (100%, 80%, 60%, 40% field capacity). Growth rate of *L. japonicus* and *L. burttii* was slow, whereas *L. filicaulis* was closer to cultivated species in terms of foliar and root development. There was no conclusive identification of water tolerant and sensitive genotypes or tap-root and branched-root system with model species.

3.5. Measurements of water use efficiency

Water use efficiency (WUE) is a physiological marker that has provided information about hydric deficit tolerance in several species such as rice, wheat, sorghum, barley and bean. Thus, this parameter can be used as functional marker of hydric deficit to support breeding programs as well as give a better comprehension of plant response to drought. Considering that plant dry matter production is mainly related with soil water availability, it is possible breeding genotypes for drought tolerance on the basis of a higher WUE, since WUE is the relation between plant dry matter accumulated and transpired water. This parameter can be analyzed by a gravimetric methods, wherein the water consumed is weighted and referred to the total biomass at the end of the assay, or can also be estimated by the analysis in the tissues of $^{13}C/^{12}C$ ratio.

Lotus model species and cultivated ones have WUE distinctiveness when plants are subjected to hydric deficit conditions (50 % or less of water field capacity) (Figure 7).

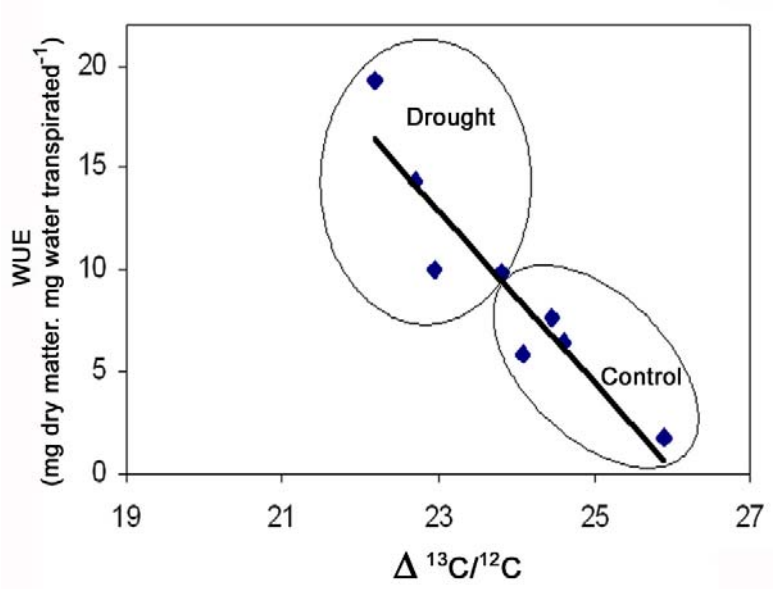

Figure 7. Illustrative relationship between $\Delta^{13}C$ and WUE in different genotypes under well-watered (control) and drought stressed conditions.

WUE is closely related with plant capacity to control opening and closing stomata, a parameter called stomata conductance (SE) that can be estimated by a porometer. *L. corniculatus* cv. San Gabriel has a higher SE than both cultivars of the model species *L. japonicus* Gifu and MG20 when no water restrictions are present, and this may cause a higher biomass production in the cultivated species. At the same time, *L. corniculatus* has a faster stomata closing in response to hydric deficit (Figure 5), which means that this species has a better control of water losses. SE in *Lotus* had a problem of reproducibility which means that it can not be used as a good drought tolerance marker in *Lotus* breeding, but it is a nice complement to study and understand the WUE.

WUE can be also estimated by the isotopic discrimination ratio $^{13}C/^{12}C$, based in the property of Rubisco to discriminate differentially ^{12}C against ^{13}C. Results of the analysis of WUE quantified by $^{13}C/^{12}C$ allowed us ranking the species and cultivars of the *Lotus* genus as shown in Figure 5.

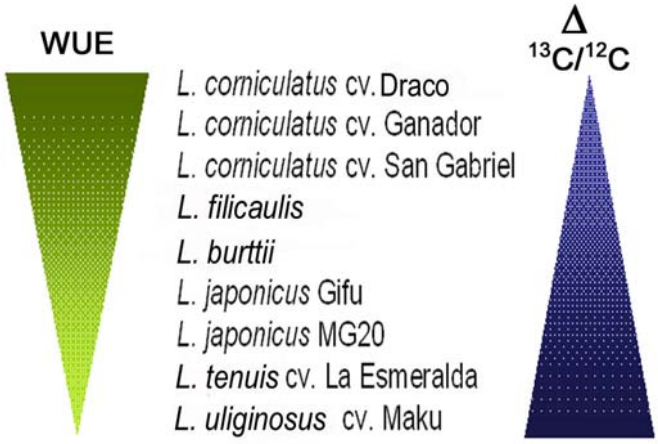

Figure 8. Ranking list of *Lotus* sp. genotypes according WUE and Δ^{13}. Genotypes were listed from the best WUE on the top to worst WUE on the bottom.

Interestingly, *L. corniculatus* cv. INIA Draco, a genotype characterized by its high persistence under field conditions, showed better WUE, estimated by this methodology, than *L. uliginosus* cv. Grasslands Maku which in field conditions presents a rapid dehydration and wilting phenotype. In this last species, development of rhizomes is probably a strategy to survive the periods of drought, since that tissue allows the plants to re-grow once the drought has passed.

Studies with different *Lotus* species validate the carbon isotopic discrimination ($\Delta^{13}C/^{12}C$) technique as the best parameter to establish physiological differences amongst genotypes in relation to water use efficiency under well watered or drought conditions**.**

3.6. Alteration of photosynthesis during drought conditions

Photosystem II (PSII) functionality by measurement of PSII chlorophyll fluorescence is a typical parameter that has provided information about hydric deficit tolerance in several species such as spinach, *Arabidopsis* and *Stypa*. Chlorophyll fluorescence emission is easily measurable by the use of a portable equipment, and can provide quantitative and qualitative information about the photosynthetic functions to evaluate the physiological status of the plants.

PSII analysis through the ratio Fv/Fm can only be used as a functional marker of hydric deficit response in *L. corniculatus* and *L. japonicus* when temperature of 40 ºC is applied simultaneously to the drought situation. A decrease in the ratio Fv/Fm was detected in both species (Figure 9). This stress condition is similar to the one occurring during drought period in summer season in the field, and Fv/Fm can then be used as functional marker of drought stress. On the other hand, *L. uliginosus* plants were the most sensitive to drought stress, showing a reduction of 50 % in the ratio Fv/Fm after 7 days of hydric deficit. This result indicates substantial damage produced in the photosystems as result of drought, which were also accompanied by clear symptoms of wilting. PSII stability under hydric deficit conditions in this genus is therefore an interesting topic to be further studied in detail, because species-dependent efficient protection mechanisms are likely involved and should be explored more deeply.

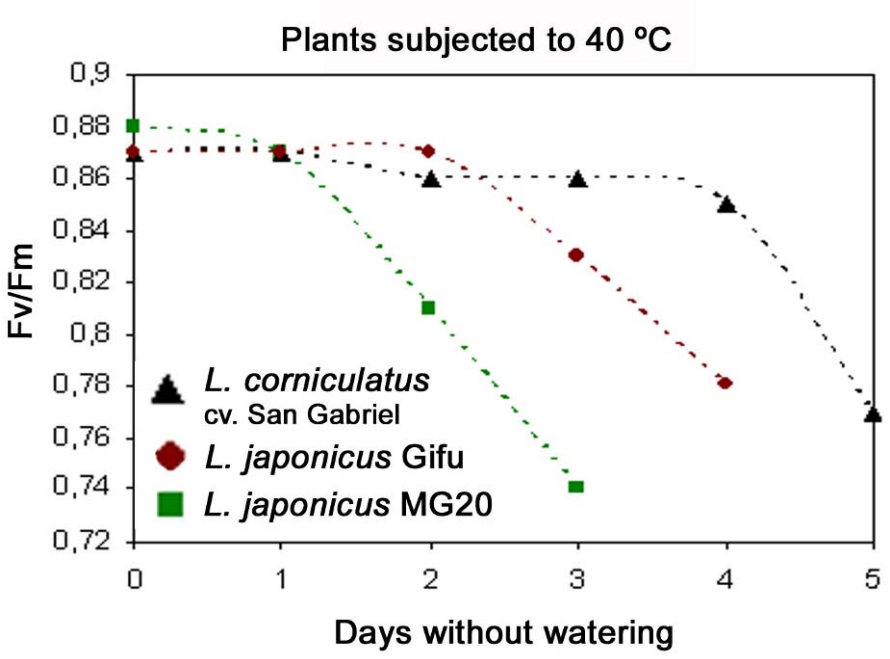

Figure 9. Changes in Fv/Fm associated with drought in *Lotus corniculatus* and *L. japonicus.*

Chlorophyll thermoluminiscence (TL) and high temperature thermoluminiscence (HTL) were also shown to be very valuable techniques for the analysis of photosynthesis in the drought stress response of *Lotus*. A curious singularity of TL bands was observed in *Lotus japonicus* Gifu showing a very high AG/B ratio which is indicative of a peculiar high level of photosynthetic assimilatory power in this plant in control conditions, possibly related with the fact that this plant is a root nitrate assimilator. This pattern of AG and B bands is substantially altered by drought, thus constituting another photosynthesis parameter related with drought in this plant. Furthermore, a characteristic band of HTL indicates a high level of lipid peroxidation in *Lotus*.

No clear trends related to drought were detected in *Lotus* for enzymes of photorespiration (some of them increase, some of them decrease), neither in the photorespiration rate or amino acids involved in photorespiratory metabolism.

3.7. Proline accumulation in response to drought stress

Proline accumulation by de novo synthesis is a common response to different types of stress situations in a wide variety of organisms. Multiple protective functions have been assigned for proline that may explain this conserved response during stress (compatible osmolite; C-N storage for recovery after drought; Reactive Oxygen Species protectant - mainly hydroxyl radicals scavenger; protectant of macromolecules and membranes; component of stress important proteins; cellular homeostasis; redox potential regulation; control of gene expression, etc.).

In *Lotus,* both the model (*L. japonicus*, *L. burttii* and *L. filicaulis*) and the agronomic important species (*L. corniculatus*, *L. tenuis* and *L. uliginosus*) accumulate proline in response to drought. Thus, proline showed to be a very nice marker of drought stress in *Lotus*, and a strong correlation was detected between proline concentration and hydric deficit. In these species proline accumulation begins at early stages of drought imposition, when water content variations in the tissues are very low. As low as 10 % decreases in the relative water content are enough to trigger proline accumulation in the tissues. Therefore, proline could be considered as an early and sensitive drought stress indicator in *Lotus*, although the exact role of this proline in sensitivity-tolerance has not been yet fully defined. It has been observed that proline content is affected by the kind of the nitrogen source applied in the nutrient solution, since when *L. corniculatus* y *L. japonicus* plants are grown with ammonium the proline accumulated is at least twice than in plants grown with nitrate. On the other hand, the use of glutamine-synthetase deficient mutants from *L. japonicus* Gifu has enabled us to further investigate proline metabolic pathways and the role of proline in drought stress. These mutants were specifically affected at single point mendelian recessive mutations in the *Gln2* gene corresponding to plastidic glutamine synthetase. Lower accumulation of proline detected in these mutants was correlated with a worst rehydration ability of mutant plants after drought but do not have any effect on the rate of water loss during the stress. The results argue against a role of proline as a compatible osmolite for the drought stress response in this plant, but suggest a role of this metabolite in the recovery of the plant after drought stress. In addition, an important alteration of proline metabolic pathways was detected also in mutant plants, suggesting a control of these pathways by the level of proline that is accumulated in the plant. Furthermore, the lack of plastidic glutamine synthetase makes these mutants unable to reassimilate ammonia released in

mitochondria as a result of the C-N photorespiratory cycle, reason why these mutants show a dramatic air-sensitive (photorespiration) phenotype (Figure 10).

Figure 10. Air-sensitive phenotype of plastidic glutamine synthetase mutants used in this work. Mutant plants are healthy in a CO_2-enriched (photorespiration-suppressed) atmosphere but show chlorosis and necrosis symptoms when transferred to ordinary air (active photorespiration) conditions.

Our results show that plastid glutamine synthetase is clearly involved in drought stress response, and therefore there is a clear interconnection between N-assimilation, photorespiration and drought stress, confirming our working hypothesis at the beginning of the project. Important alteration was detected in the whole mutant plant transcriptome (see below), including many stress-responsive as well as photosynthesis related genes.

3.8. Transcriptomic and metabolomic changes during drought stress in *Lotus*

New functional genomic tools were applied to further increase our knowledge of *Lotus* response to drought stress, and further defining putative molecular markers. For this purpose, a set of different affychips from Affymetrix (Figure 11) were used to carry out the transcriptomics of drought stress.

Figure 11. The use of affychips from affymetrix for transcriptomics studies of drought stress. The image of one of the affychips used is shown on the left, together with the process of labeling for transcriptomics on the right.

These DNA chips include a total of 48,000 oligonucleotide probes for most of the genes known to be expressed in *Lotus japonicus* Gifu, and were hybridized with RNA extracted from leaves of drought-stressed (RWC around 65 %) and control plants, in order to determine the number of genes whose expression is affected by drought. 3950 probesets were found to be modulated by drought using a false discovery rate (FDR) of 5% (FDR $p < 0.05$). Of these, 2064 were upregulated and 1886 were downregulated. Among the top upregulated probes several late embryogenesis abundant (LEA) proteins were found, thus confirming the validity of the results obtained, since this large family of proteins are known to be associated with the tolerance to water stress, although its role is still not well understood. Other highly upregulated genes were associated to redox metabolism like protein disulfide isomerase, glutathione-S-transferase and several oxidoreductases, indicating that drought stress in *Lotus* is associated to oxidative stress. The different drought-elicited probes were clustered according to their relative metabolic pathways (Figure 12).

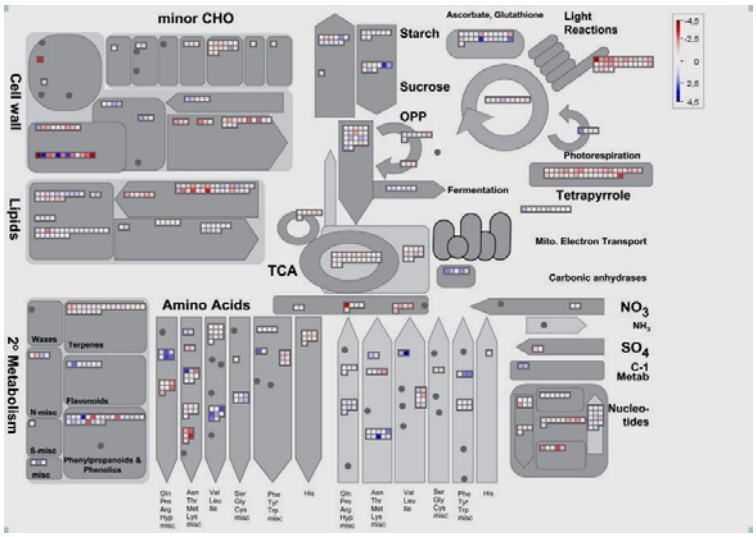

Figure 12. Mapman sketch of metabolic pathways altered in *L. japonicus* Gifu in response to drought. Each square correspond to a particular gene affected, as deduced from transcriptomic analysis. Red and blue indicate, respectively, higher and lower expression according to the scale bar on the right.

Photosynthesis and photorespiration were pathways greatly affected by drought, both downregulated thus confirming our previous results of chlorophyll fluorescence and thermoluminiscence. Drought also stimulated the transcription of the biosynthetic pathways for ethylene, ABA and jasmonic acid. These probes are thus good markers of drought in *L. japonicus* Gifu, and were found also altered by drought stress in a higher extent in glutamine synthetase-deficient mutants, as mentioned above.

Other results of LOTASSA project analyzed the possible changes in metabolite profiling of different *Lotus* species in response to drought, making use of GC-MS metabolomic facilities available. With the aim to explore adaptive metabolic mechanisms to water deficit and provide potential traits for engineering metabolic pathways in legumes, we first subjected a model plant such as *L. japonicus* Gifu to increased drought conditions in order to recognize stress-dose related changes. We further subjected the various *Lotus* species described previously to long-term water stress to compare their acclimation metabolic phenotype. The physiological responses were recorded and their metabolomic changes were non-targeted profiled with state-of-

the-art metabolomic technologies. As a whole, a certain degree of conservation in the qualitative drought-induced changes was observed but also interesting differences among drought tolerant and sensitive *Lotus* species. From the quantitative point of view, exemplified metabolites highlighted that drought-elicited quantitative changes were rather specific for each genotype. In addition, we performed a comparison of the metabolic changes from *Lotus* species under drought and salinity acclimation, with the aim to recognized shared or distinctive markers for these constraints. Interestingly, some molecules previously recognized in *L. japonicus* as non-responsive or down-regulated under salt stress, were induced under drought, while others known to be salt responsive did not change in water limited plants.

Chapter 4. Responses to salt stress in *Lotus* spp.

4.1. Introduction

Salinity in the soil and irrigation water is an environmental problem and a major constraint for crop productivity. Currently, *c.* 20% of the world's cultivated land is affected by salinity, which results in the loss of *c.* 50% of agricultural yield. Salt stress has both, osmotic and toxic effects on plants, impairing growth, ion homeostasis, photosynthesis and nitrogen fixation. The relative contribution of these two effects to the decrease in the plant's overall performance varies with the intensity and duration of salt stress, as well as with the plant species, cultivar and tissue.

In LOTASSA project, *Lotus* model and cultivated species were evaluated under short and long term salinization protocols and analysing diferents ontogenic stages. "Traditional" osmolytes used to describe the "salinity status stress" in glycophytes were investigated. In addition, salt induced changes in leaf and shoot anatomy of model and cultivated *Lotus* species were analysed (Figure 13a and 13b).

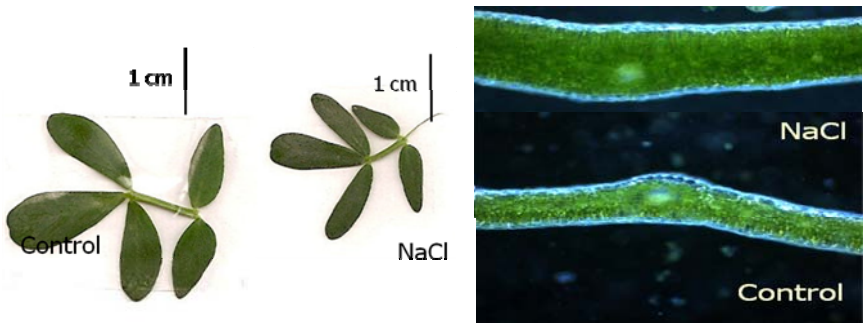

Figure 13a. Leaflet size of *Lotus tenuis* with NaCl and control conditions. 13b. Leaflet lamina

Special evaluation was performed on reactive oxygen species (ROS) tisular levels and their regulation. This analysis was included since several studies describing that oxidative stress generated by salinity, may modulate some of the toxic effects originated by ClNa. Thus, under optimal growth conditions, antioxidant defences are able to cope with ROS, whereas in plants exposed to salinity or other types of stressful conditions, the antioxidant capacity may be overwhelmed by ROS. A tight control of ROS concentrations by antioxidants is critical to prevent oxidative damage in cells while simultaneously allowing ROS to perform useful functions as signal molecules.

Simultaneosly, as was described previosuly, salinity enhances osmolites (polyamines, proline, etc) accumulation in glycophytes and there are a lot of reports suggesting that the tolerance is well correlated with high tisular levels in plants. However, salinity include no only fine ROS levels regulation and osmolites accumulation, but also include ion exclusion, ion sequestration and tissue tolerance to accumulated salts, tight control of water homeostasis and osmotic adjustment, changes in growth and development, and a wide array of underlying biochemical and molecular changes.

4.2. Antioxidant Enzymatic defences

The antioxidant defences of plants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and the four enzymes of the ascorbate-glutathione

cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase, dehydroascorbate reductase (DR) and glutathione reductase (GR) (Figure 14).

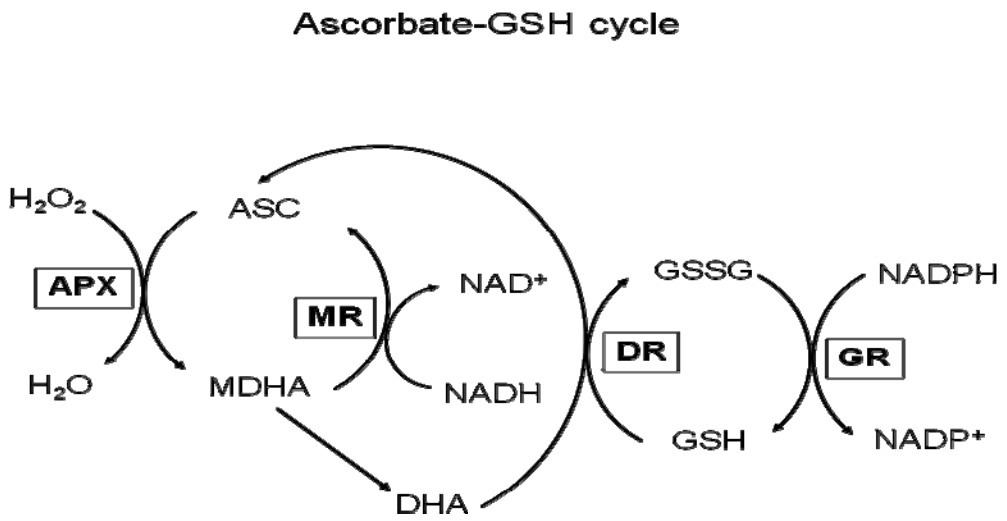

Figure 14. Ascobate-glutathion cycle.

This cycle is fueled up by ascorbate and glutathione, which are major redox compounds. In legumes like *L. japonicus*, the thiol tripeptide homoglutathione, which is a structural homologue of glutathione, may completely replace glutathione. To gain a deeper insight into the protective role of antioxidants during salt stress, plants of *L. japonicus* under two salinization protocols were evaluated: one causing only osmotic effects (*S1*) and the other having also toxic effects due to Na^+ accumulation (*S2*). Then, several physiological and biochemical markers related to salt stress were measured and the expression of encoding antioxidant enzymes was analysed. Under these experimental conditions, the leaf water potentials of *S1* and *S2* plants were slightly lower than those of control plants, indicating that salinity was causing a mild water stress.

The transcript levels of *SOD* genes increased in leaves of *S1* plants. Interestingly, the *GPX1* and *GPX6* genes, which putatively encode chloroplastic GPX isoforms, were up-regulated in the leaves of plants exposed to the two salt treatments. By contrast, could not detect any change in the mRNA levels of *GPX* in the roots.

The concentrations of total ascorbate and homoglutathione were also determined in salt-stressed plants. The total ascorbate content of leaves and roots remained unaffected with the salt treatments. Likewise, the ascorbate and homoglutathione redox states (both in reduced form) remained unchanged in leaves and roots.

The overall results obtained by Laboratory of "Estación Experimental Aula Dei (CSIC-Spain) sugested that *L. japonicus* ecotype MG-20 is more tolerant to salt stress than other legumes, which can be attributed to the plant's capacity to avoid Na^+ reaching the shoot and to activate antioxidant defences.

In addition, the IFFIVE group (INTA-Argentine) evaluated the phenotyping of four *L. japonicus* (MG-20 and Gifu ecotypes) as well *L. burttii* and *L. filicaulis*, using two NaCl levels (50 and 150 mM) applied by acclimation during 28 or 60 days, and evaluating growth and related parameters, such as photosynthesis, ions, relative water content, oxidative damage and antioxidant system responses. Growth responses in the four *Lotus* genotypes varied according to plant developmental stage. *L. japonicus* MG-

20 appeared as salt-tolerant genotype, mainly when salt stress occurred during the young plant stage. The capacity of *L. japonicus* MG-20 to sustain growth under salt stress correlated with enhancement of SOD and GR activities. Moderate salt treatment of 50 mM applied to this ecoytype in short or long term assay did not affect either biomass production or photosynthesis.

Transgenic *L. japonicus* MG-20 overexpressing Mn-Superoxide dismutase (T-SOD) and Glutathione Reductase (T-GR) were obtained, and the transgenic lines showed higher total SOD and GR activities in comparison with WT under control conditions. Likewise, T-GR also exhibited higher reduced glutathione content. Lipid peroxidation increased under 150 mM NaCl in transgenic and non transgenic lines, but WT showed higher values. T-GR showed significantly higher relative water content under salt stress; however, T-SOD plants had a significantly better water use efficiency, suggesting pleiotropic effects due to antioxidant enzymes overexpression that affected water metabolism. Interestingly, number and nodule weight were higher in T-SOD under 50 mM treatment, whereas at 150 mM T-GR plants showed much higher values, with significant differences respect to T-SOD and WT lines. Nitrogen fixation were also higher in transgenic lines under 50 mM salt stress.

Finally, a positive correlation between apoplastic ROS production in roots with plant growth and nodulation was observed in WT plants. Even though apoplastic ROS production in transgenic roots was lower than in wild type under control conditions, transgenic plants maintained a little ROS production under 150 mM NaCl, whereas in WT this was abolished.

4.3. Proline and polyamine accumulation

Salinity enhanced proline levels in all species (model and crops *Lotus* species), and significant differences in proline accumulation between *L. japonicus* ecotypes were also observed. In the same way, 27 *L. tenuis* varieties/accesions collected by INTA Pergamino in the Salado River Basin, Argentine, (Figure 15) showed increased levels of this aminoacid and the differences between them being evident, both, under control and stress conditions. These accesions were previously clasified as “tolerant” or “sensitive” after comapring their relative growth under control and saline stress in presence of 150 mM ClNa (Figure 16).

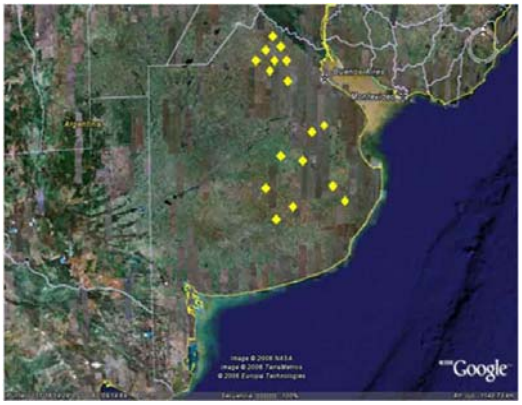

Figure 15. Collection sites of *Lotus tenuis* in Buenos Aires Province, Argentine.

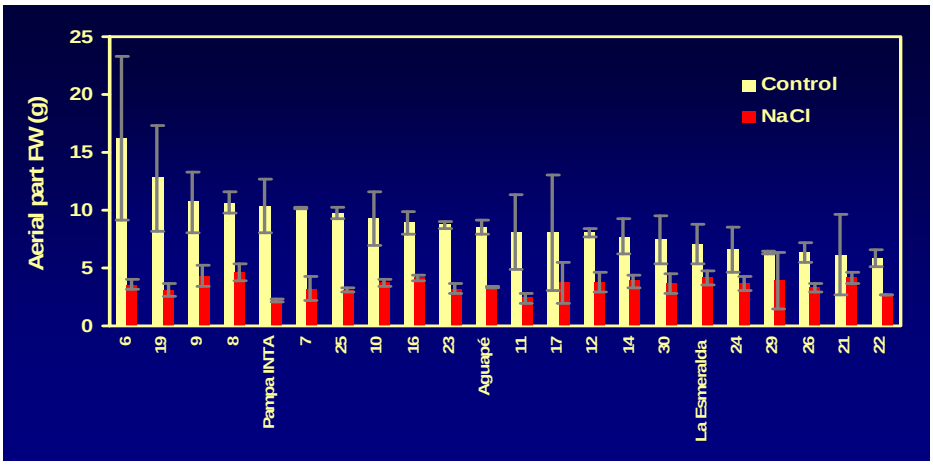

Figure 16. Growth of accessions/cultivars of *Lotus tenuis* with and without NaCl.

A typical display of the Na^+ tisular concentration and morphological alterations observed simulteously in the diferents acesions under saline stress is displayed in Figures 17a, 17b and 18.

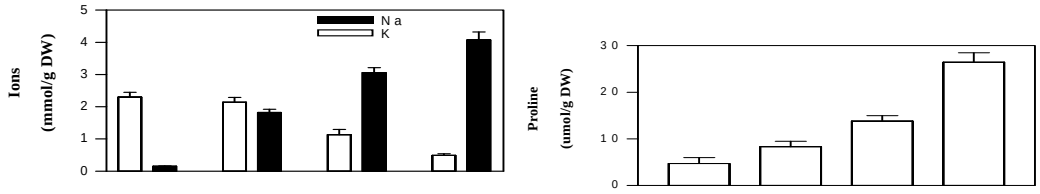

Figure 17a. Ions concentration of accessions/cultivars of *Lotus tenuis* with and without NaCl. 17b. Increase of proline concentration with the addition of NaCl.

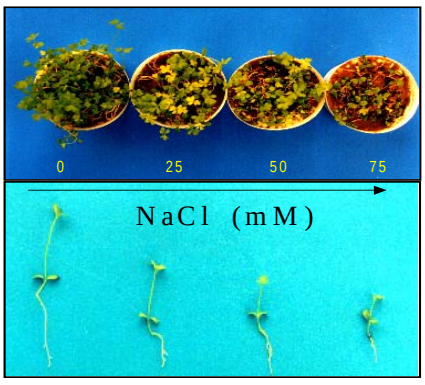

Figure 18. Reduction of *Lotus tenuis* growth with increased concentrations of NaCl

Under identical experimental conditions, changes in free polyamine accumulation associated to saline stress were evaluated in model and several *Lotus tenuis* accessions, showing as a general rule a decrease in the spermidine/spermine

(spd/spm) ratio (Figure 19). No changes were observed in putrescine concentrations provoked by salinity.

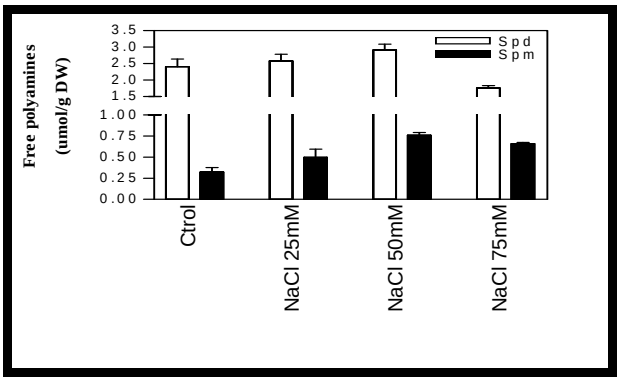

Figure 19. Reduction of free polyamine accumulation with NaCl

However, no clear correlations can be stablished between salt-induced changes in proline and polyamine levels of *Lotus tenuis* accesions, comparing diferent ontogenic status and diferent experimental protocols, sugesting that these parameters would be not useful as biochemical markers of salt tolerance.

4.4. Comparative functional genomics of salt acclimation in *Lotus* spp. (model and cultivated plant species)

Bearing in mind that plant research and breeding efforts are underway to identify genes and processes involved in salt stress responses and to produce new crop varieties with greater salt tolerance, the Max Planck Institute for Molecular Plant Physiology group of LOTASSA consortium, carried out a comparative functional genomic study of three model and three forage species of the legume genus *Lotus*, representing more sensitive and tolerant genotypes. The relative salt tolerance of the seven *Lotus* genotypes (including G-20 and Gifu ecotypes of *L. japonicus*) was determined in two independent experiments that subjected plants to long-term step-wise increases in the level of NaCl, up to a lethal concentration of 300 mM NaCl.

Significant differences were observed between diferent genotypes, and of the more sensitives began to die within a few days of reaching 300 mM NaCl in the watering solution at day 28, while plants of the most tolerant ones did not begin to die until after day 60 (Figure 20).

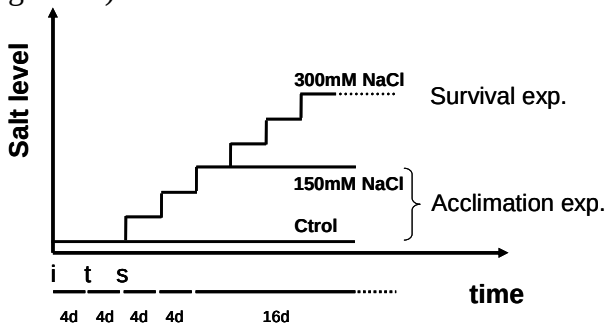

Figure 20. Reduction of plant survival with increased concentrations of NaCl with time

Under these experimental conditions, they defined the 'lethal-dose fifty' (LD50) as the number of days at which more than 50 % of plants had died. The relative tolerance observed was: *L. glaber* > *L. burttii* > *L. japonicus* var. MG20 > *L. filicaulis* > *L. japonicus* var. Gifu ~ *L. uliginosus* ~ *L. corniculatus*. Interestingly, the difference in tolerance between the two genotypes of *L. japonicus* was of the same magnitude than that between most other sensitive-tolerant pairs.

The second salt treatment regime (150 mM NaCl) was applied to facilitate comparison of salt acclimation responses between the different species under physiologically meaningful conditions (Figure 21).

Figure 21. Trays of *Lotus japonicus* growing in media with different NaCl regimes.

In these experiments, shoot Na^+ and Cl^- concentrations increased dramatically in all cultivars in response to the treatment, while K^+ concentration changed less in the models than in the forage species. Other interesting observation was that salt accumulation in the stress-acclimated genotypes exhibited a negative linear correlation with LD50 under lethal salinity, with a better correlation coefficient for Cl^- than for Na^+ levels. These results are in line with previous physiological observations demonstrating that more tolerant glycophytes display decreased salt accumulation compared to sensitive ones.

Reinforcing the critical importance of the salinization protocol and ontogenic status, significant induced changes in macro- and micro-nutrients in the sub-lethal salt acclimation experiments were profiled using ICP-AES and analyzed by ANOVA. These evaluations revealed variability at the nutritional level between the *Lotus* cultivars in response to salt stress. The results obtained suggested that nutritional aspects were differentially altered under salinity between more sensitive and tolerant backgrounds, regardless of them representing model or forage legumes.

Overall, the data showed a reasonably good match between in-breeding model species and out-breeding crop species in their range of tolerance, salt accumulation, induced growth changes and variation of nutrient content.

To compare genome-wide transcriptional responses to salt stress of the different genotypes, the Max Planck group performed sub-lethal NaCl-acclimation experiments and analyzed them using the Affymetrix GeneChip® *Lotus* Genome Array. Of the total probe-sets that detected transcripts in all cultivars, more than 50% showed significant changes in transcript abundance in at least one genotype upon salt acclimation.

Interestingly, transcript levels of less than 1% were significantly altered in all seven genotypes and the up- and down- regulated transcripts shared by sensitive and tolerant genotypes included a set of genes already known to be involved in general plant stress responses. The results obtained suggested that not only qualitative but also quantitative transcriptional differences were involved in tolerance.

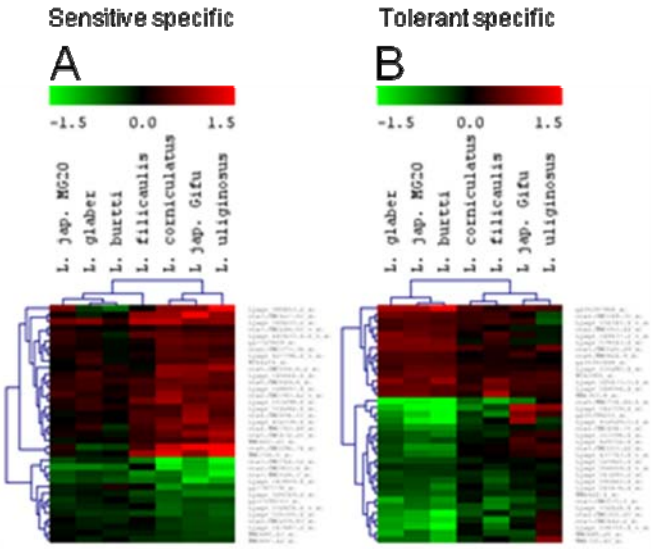

Figure 22. Transcriptomic analyses of *Lotus* spp. under salt stress.

Accordingly, the relative change in gene expression of many of the sensitive- and tolerant-specific transcripts did not correlat well with biomass but with the ion content of all genotypes under stress, particularly with Cl^-. Moreover, the results of the transcriptome analysis indicated that only a small fraction of the transcriptional responses to salt stress were conserved amongst the *Lotus* genotypes.

Simultaneously, the metabolic phenotypes of the *Lotus* cultivars subjected to sub-lethal salt treatments were determined using non-targeted GC/EI-TOF-MS. One hundred and three analytes were identified, representing both known and unknown compounds. All of them were present in all genotypes and in all three independent experiments. Many NaCl-induced changes in metabolite levels were qualitatively similar in some or all genetic backgrounds, although some genotype-specific metabolic changes were evident. However, in general, metabolites did not show good correlation coefficients when correlated with ions or biomass under stress, probably reflecting the inherent biological variability of metabolic networks and/or distinct quantitative changes between the *Lotus* genotypes. Taken together, the results from the metabolomic analyses indicated that some but not all of the responses of primary metabolism to salt stress are conserved between *Lotus* species, as metabolic network properties displayed both common and genotype-specific interactions. The majority of the metabolites that accumulated in model plants under salinity were specific for a genotype or shared with other models, whereas those down-regulated were mostly shared by several genotypes.

4.5. Concluding remarks

The comparative morphologial, physiological, ionomic, transcriptomic and metabolomic analyses of salt-stressed *Lotus* genotypes revealed the existence of conserved and non-conserved responses to salinity within this genus. Moreover, the

physiological experiments performed by LOTASSA consortium groups showed that the model plants are appropriate tools to study salt acclimation and tolerance in this group of legumes.

Chapter 5. Low pH and Aluminum Stress

Aluminum (Al) toxicity is a major constraint to crop yields in acid soils. Thus, at soil pH≤5 Al is solubilized into the soil solution, inhibiting root growth and function. Forage legumes play an important role in the productivity of cultivated pastures, due to their ability for nitrogen fixation and growth in nutrient-poor conditions. The *Lotus* species have a good potential in cultivated pastures, which is related to their ability to grow in slightly acid soils, and their moderate tolerance to aluminum, manganese and sodium stresses. *Lotus* species are planted mainly in South and North America, some countries of Europe and Australia. Among the genus, *L. corniculatus* is undoubtedly the species considered to have the greatest agricultural importance and the widest distribution.

The effects of Al on antioxidant genes and metabolites in roots and leaves of *Lotus corniculatus* cv. INIA Draco were investigated by CSIC Aula Dei, a forage legume of agronomic interest which is related to the model legume *L. japonicus*. For this purpose, seeds were germinated and grown in agar plates for 9 days, then transferred to hydroponic cultures containing 200 μM$CaCl_2$ and 0, 10 or 20 μM $AlCl_3$. Plants grown in the presence of 10 μM Al showed reductions in shoot fresh weight, root fresh weight, leaf area and root length. The corresponding decreases with 20 μM Al were 73%, 78%, 64% and 52%, respectively. The expression (mRNA) levels of thirty antioxidant enzymes and two Al-activated malate transporters (ALMT) were determined in roots by real-time quantitative PCR. Treatment of plants with 10 μM Al for 14 days caused up-regulation of cytosolic Fe-superoxide dismutase and down-regulation of plastidial Fe-superoxide dismutase. Metabolic profiling of roots and leaves was performed in derivatized extracts using gas chromatography-mass spectrometry according to established protocols. This analysis showed that the treatment with 10 or 20 μM Al increased the contents of some amino acids (asparagine, glycine), sugars (glucose, fructose), polyols (pinitol) and organic acids (succinic acid, threonic acid, 2-methylmalic acid, 2-isopropylmalic acid) in roots. Some of these metabolites, such as asparagine and 2-isopropylmalic acid, were also increased in the leaves. In particular, the increases of 98% and 61% in 2-isopropylmalic acid in roots and leaves, respectively, with 20 μM Al may reflect the plant's need to synthesize Al-chelating compounds. This rather unusual organic acid was previously found to be secreted by yeast cells in response to Al. Based on these studies, it is proposed that 2-isopropylmalic acid is a major Al-detoxifying organic acid in *L. corniculatus*.

Some mechanisms for detoxification of other metals using biochemical and molecular biology approaches in *L. japonicus* were also investigated. One such mechanisms involves metal chelation by phytochelatins. These are cysteine-rich polypeptides that bind some metals with high affinity. The resulting metal-phytochelatin complexes are stored and degraded in the vacuoles, thus avoiding the toxic effects of metals on cellular metabolism. Phytochelatins are synthesized by the enzyme phytochelatin synthase (PCS) using glutathione as a substrate. The synthesis of phytochelatins in plants after treatment with cadmium (Cd) and other metals, including Al, was studied. After addition of these metals, roots started to synthesize elevated amounts of phytochelatins, indicating the presence of active *PCS* genes. Three genes could be identified (*LjPCS1*, *LjPCS2* and *LjPCS3*) and were mapped on chromosome 1. The genes differ in exon-intron composition and responsiveness to Cd. Gene structures and phylogenetic analysis of the three protein products are consistent with two

sequential gene duplication events during evolution of vascular plants. It was found that whereas *LjPCS1* is similar to other plant proteins with PCS activity, *LjPCS2* and *LjPCS3* are rather unusual. These proteins are much shorter and their encoding RNAs show alternative splicing; however, they still conferred tolerance to heavy metals when expressed in yeast cells. In particular, *LjPCS3* was activated by Al, whereas *LjPCS1* was not. These results unveil complex regulatory mechanisms of PCS expression in legume tissues in response to heavy metals and Al.

Some metals induce oxidative stress in plants, therefore the effect of Al on glutathione peroxidases (GPXs) was examined (Figure 23). These are antioxidant enzymes that catalyze the reduction of phospholipid hydroperoxides in biological membranes and hence protect them from oxidative damage. Six *GPX* genes have been identified in *L. japonicus* and *L. corniculatus*. Plants of *L. corniculatus* were exposed to 20 μM Al for 1-24 h. Treatment with Al decreased expression of all *GPX* genes, which suggests that the toxic effect of this metal is in part due to a decrease in antioxidant defences leading to peroxidation of membrane lipids.

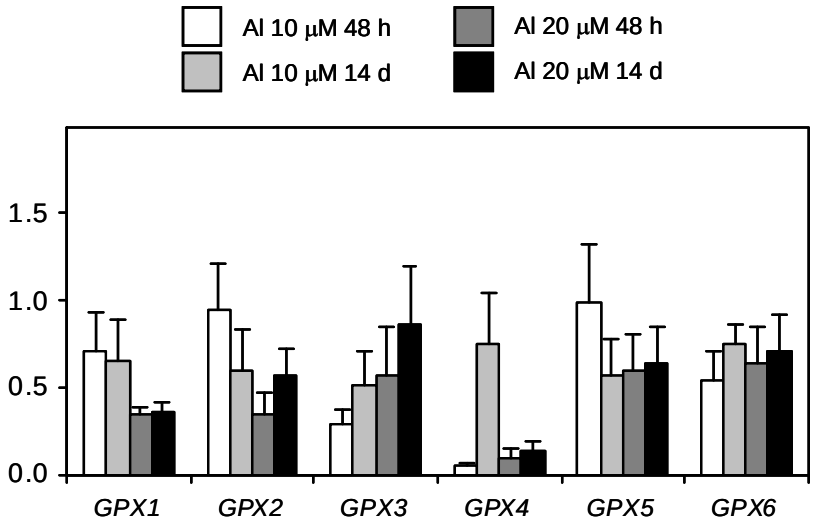

Figure 23. Effects of aluminium on the expression of genes encoding glutathione peroxidases (GPXs) in roots of *Lotus corniculatus*. Values of mRNA levels were normalized relative to *ubiquitin* and expressed relative to those of control (untreated) plants.

Using electrophysiological measurements it is possible to define motive forces for ions at the plasma membrane. Several agriculturally important cultivars of *Lotus corniculatus* were tested for growth and membrane potential values (Em) and a good correlation was found between them under Al stress (Botanický ústav SAV, Slovakia). The decrease of Em was caused more by the decrease of diffusion potential (Ed) than by the active part of the potential generated by the H^+-ATPase activity (Table 2). The drop of Ed under Al stress is accompanied by the significantly higher K^+ loss from the root tissue in the more sensitive cultivars. From these results, INIA Draco was chosen for further experiments as a relatively sensitive cultivar and UFRGS was chosen as a tolerant one.

Since root elongation is associated via H^+-ATPase activity localized at the plasma membrane, their activity was investigated during Al treatment using fusicoccin (FC) and measured an increase of O_2 consumption. Al caused just slight decrease of range and velocity of the membrane hyperpolarisaton in UFRGS cultivar, whereas in

INIA Draco much higher inhibition was observed. These results suggest the better ability of the cultivar UFRGS to maintain the function of the ATP pumps under Al stress than in the case of cultivar INIA Draco. Al-tolerant UFRGS had also significantly lower rate of O_2 consumption than Al-sensitive INIA Draco.

Table 2. Resting potential (E_M) and diffusion potential (E_D) after 4-day treatment with pH=5.5, pH=4.0 and pH=4.0 + 2 mM $AlCl_3$ (±SE, n=25).

cultivar	*pH=5.5*		*pH=4.0*		*pH=4.0 + 2mM Al*	
	E_M	E_D	E_M	E_D	E_M	E_D
INIA Draco	133.6 (±1.2)	72.5 (±1.2)	121.4 (±1.6)	68.0 (±0.8)	86.4 (±1.6)	31.4 (±1.3)
Est. Ganador	128.0 (±1.2)	72.5 (±0.6)	117.3 (±1.1)	68.9 (±0.6)	100.1 (±0.7)	49.8 (±0.5)
San Gabriel	125.6 (±0.8)	72.6 (±0.8)	113.0 (±1.1)	67.3 (±0.9)	101.8 (±1.3)	40.2 (±1.2)
São Gabriel	126.4 (±1.5)	72.6 (±0.9)	122.3 (±1.0)	70.3 (±0.8)	105.8 (±1.2)	50.8 (±1.0)
UFRGS	131.9 (±2.4)	72.2 (±1.1)	131.0 (±1.2)	72.0 (±0.8)	110.4 (±1.8)	54.4 (±0.7)

Light and electronic microscopy showed increased vacuolation in root tips after low pH treatment (pH=4.0), small vacuoles occurred in centripetal cell layers. The treatment with Al caused damage of several epidermal cells, with disintegrated structure. The cortical cells were strongly vacuolated close to the root tip. After Al treatment, huge cell wall protuberances were visible and the cells lacking such wall modifications were damaged more severely, suggesting their protective role. The experiments showed that the response to pH as well as to Al treatment was similar in the model plant, *L. japonicus* Gifu and in *L. corniculatus* the differences between the cultivars were rather quantitative. The more severe damage of epidermal cells and first cortical layers (disintegrated nucleus and all cytoplasmic organelles) was found in cultivar INIA Draco, in which the structural injury was also observed in the more centripetally situated cortical cells.

The characterization of the germplasm available (diploid and tetraploid) for Al tolerance and the selection of populations with better performance under soils with a high Al saturation was also performed by UFRGS (Porto Alegre, Brazil). The first step was the characterization of *Lotus* species in soil (Figure 24) and also in nutrient solution (Figure 25). The presence of Al caused a strong growth reduction in all plants (Figure 26). Among the model species, *L. japonicus* Gifu was the best under stress conditions (Figure 27).

Figure 24. Characterización of the effects of aluminium on *Lotus* species in soil

Figure 25. Characterización of the effects of aluminium on *Lotus* species in nutrient solution

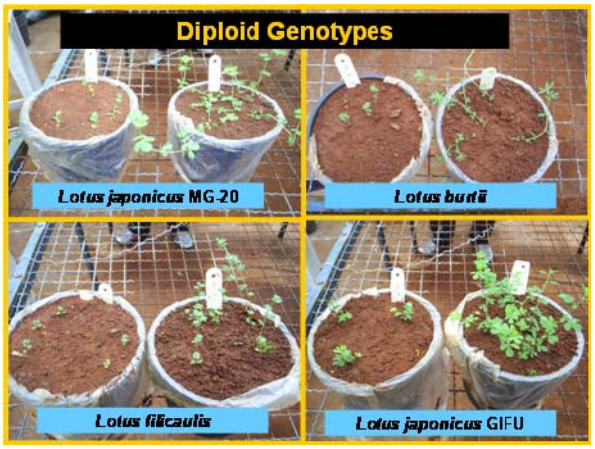

Figure 26. Effects of aluminium on all model species.

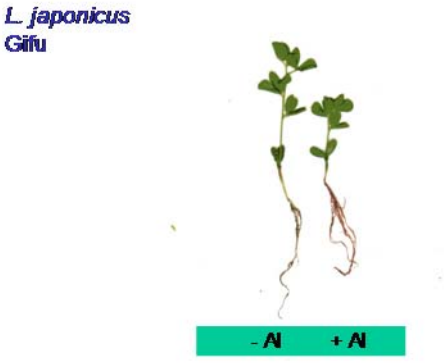

Figure 27. Effects of aluminium on *L. japonicus* Gifu under stress and control conditions.

Among the tetraploid germplasm the UFRGS and the São Gabriel populations presented the best performance under stress conditions, while alfalfa, a sensitive check had a very poor performance under these conditions, confirming the Al stress present (Figure 28).

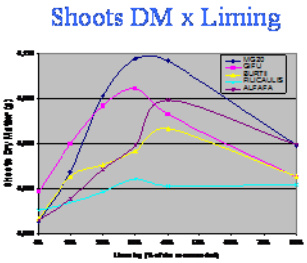

Figure 28. Performance of model *Lotus* species and alfalfa under differential Al stress in soil conditions.

Since the experimentation under soil condition is very time consuming (Figure 29), an alternative method was developed using nutrient solution (Figure 30). The results also showed a large reduction of growth caused by Al and also confirmed the differential response among the tetraploid germplasm, with UFRGS (tolerant) and INIA DRACO (sensitive) showing contrasting responses. A set of RILs (recombinant inbred lines) formed by crosses among the model plants was also tested in order to use the information already obtained with these plants to speed up the breeding process of the cultivated plants. There was a large variability among the RILs for Al tolerance, with a set of lines showing a better performance than the more tolerant parent (*L. japonicus* Gifu), pointing out to the possibility to use them in the breeding program.

Figure 29. Procedure to adjust soil Al concentrations

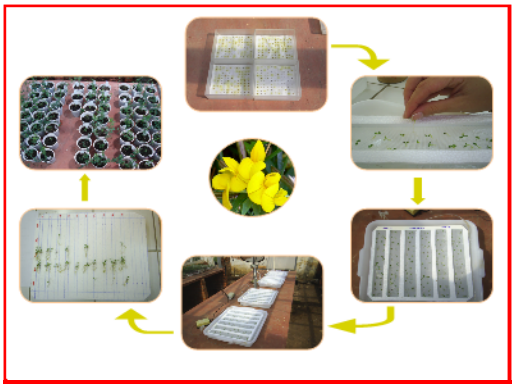

Figure 30. Procedure for the evaluation of Al tolerance with nutrient solution

After the initial characterization described above, a selection of populations with contrasting responses to Al was started, as well as for different roots types (Figure 31). Two selection cycles were performed, under soil and also under nutrient solution. The

results so far show an improvement in the selected populations when tested in soil or in nutrient solutions. The selected populations are being crossed (Figure 32) and should now be tested under field soil conditions, with the appropriated *Rhizobium* (selected in other part of the project), to evaluate the progress made. The production of organic acids by the selected germplasms was measured, since this is one of the mechanisms related to Al tolerance in plants. The original and the F_1 populations of UFRGS, INIA Draco, San Gabriel-Uruguay and São Gabriel-Brazil were submitted to a high Al concentration and the production of oxalic acid was measured. The root exudation results demonstrated that of the three organic acids tested, the only one quantified by the chromatography was oxalic acid. Of all the *Lotus* genotypes evaluated, the UFRGS population exhibited a greater oxalic acid exudation compared to the others. The protective role played out by the oxalic acid has already been demonstrated in experiments realized with other species and could be related to the tolerance observed in *L. corniculatus*. This will be very helpful in the selection of tolerant materials as well as for the explanation of the mechanism of Al tolerance involved in *L. corniculatus*.

Figure 31. Characterization of *Lotus corniculatus* root types in soil

Figure 32. Procedure for manual cross of selected phenotypes

Another species estudied by Univ. Austral (Chile) was Greater lotus (*Lotus uliginosus,* Lu), which is naturalized in Chile, mainly in the southern zones where the acid soils are predominat. Twenty-eight Lu populations, collected from different

agroecosystems in the south of Chile were characterized according to their sensitivity/tolerance to Al stress under field conditions. Plants were grown in soils under three exchangeable Al levels to evaluate their performance and to select plants sensitive and tolerant to Al. The individual selection criteria were plant height, number of leaves and number of branches per plant. The plants selected as tolerant were the ones with higher scores in 0.5 and 1.0 cmol kg^{-1} Al, and the sensitive those with lower scores in 0.5 cmol kg^{-1} Al. Sensitive plants had in average 67% lower height, 40% lower number of leaves and 30% lower number of branches than the tolerant ones. The plants selected were transplanted to a condition without Al to continue growing until the production of seeds. For this they were grown separately and cross pollinated within each group of Al sensitivity/tolerance. Additionally, the plants were kept growing for the whole season and then harvested to evaluate their dry matter production (shoot and root biomass) as population average, root length and density and Al concentration both in above ground material and roots. The populations were classified in four categories according to their tolerance or sensitivity to Al. The populations obtained from the previous selection (two tolerant and two sensitive) and the species considered as model plants were evaluated to compare their performance under five levels of Al in solution at pH 4.2, in hydroponic culture. The results suggested different mechanisms of adaptation and tolerance to the phytotoxic effects of Al. The populations classified as tolerant in field trials maintained this feature. *L. japonicus* ecotype Gifu showed a greater tolerance to Al phytotoxicity compared to the other model plants. The aerial biomass, variable used as a parameter of tolerance or sensitivity of the species and populations, decreased significantly over 180 µmol Al L^{-1} in the tolerant populations. Nevertheless, in the sensitive populations, aerial biomass decreased significantly over 100 µmol Al L^{-1}. The root length was strongly decreased, showing the high sensitivity of this parameter to the exposure to high Al concentrations.

Another experiment to characterize the biological behaviour of greater lotus populations (two sensitive, Sen1 and Sen2 and two tolerant, Tol1 and Tol2), selected previously, compared with the model species, *L. burttii* (Lb), *L. japonicus* (Lj), ecotypes MG20 and Gifu, and *L. filicaulis* (Lf) was carried out. All the species were grown under five Al levels, pH 4.2, in growth chamber. The shoots and roots dry matter yield of all the tested genotypes decreased when Al in solution was increased. However, the dry matter reductions were different depending of the *Lotus* species or Lu populations. Lf, Lb, Lj MG20 and Lu Sen1 were the most sensitive to Al toxicity (100 µmol Al L^{-1}). Gifu and Lu Sen2 were in an intermediate position. The most tolerant genotypes to Al toxicity were the Lu selected populations Tol1 and Tol2, which tolerated up to 180 µmol Al L^{-1} and decreased sharply their dry matter yield only in 300 µmol Al L^{-1} level. All *Lotus* species had high Al concentrations in shoots and accumulated aluminum in roots. The most tolerant species accumulate more Al in roots without decreasing shoot yield.

Conclusions

Different negative effects of Al and acidity on Lotus plants were identified, such as decreased root and shoot growth, decrease of antioxidant defenses and strong damage of root epidermal and cortical cells. The selection for Al tolerance was successful, with the identification and selection of tolerant genotypes in the cultivated *Lotus* species. However, further studies need to be done in order to confirm this feature under field conditions. In addition, the accumulation and secretion of certain organic acids such as oxalic and isopropylmalic were identified as important responses and markers related to Al and acid tolerance in *Lotus*.

Chapter 6. Symbiotic nitrogen fixation by *Lotus*

6.1. Introduction

The uncertainty about the nitrogen-fixing symbiotic status of cultivated *Lotus* in some South American countries, or even the lack of information about the presence of *Lotus*-specific rhizobia in others, raises the goal of developing superior inoculants a key issue for the success of cultivated *Lotus* species in environmentally constrained soils. In some countries like Uruguay, there is long tradition in the development of inoculants for cultivated legumes. However, countries like Argentina, Brazil or Chile have little or no tradition in inoculation of forage legumes. In areas with long tradition of *Lotus* pastures, soils may contain variable amounts of rhizobia specific for certain *Lotus* species. In most cases, however, the contribution of these native or naturalised bacteria to nitrogen-fixation by *Lotus*, and therefore to forage productivity is unknown. Under optimal conditions, inoculation can lead to several-fold increase in biomass production compared to non-inoculated pastures, evidencing the agronomic, economic and ecological impact of this practise. However, particular conditions may render inoculation inefficient. A situation that commonly conditions efficiency of inoculation is related to particular soil conditions or environmental stresses for which specially adapted bacterial inoculants must be developed. Abiotic stresses affect the viability and persistence of the bacteria, and also have a strong negative impact on the process of symbiotic nitrogen-fixation.

At the start of this project, the information available about *Lotus* symbiotic rhizobia was rather limited, indicating that *Mesorhizobium loti* was the type symbiont for certain plant species like *L. tenuis*, *L. corniculatus* or *L. japonicus*, whereas other like *L. uliginosus* could only establish efficient symbioses with certain rhizobia catalogued as *Bradyrhizobium* sp. (loti) but poorly studied. Indeed, recommended inoculants for forage species were based on strains belonging to either of these two groups.

Among the objectives of Lotassa were the establishment of a collection and detailed catalogue of *Lotus* symbiotic bacteria in participating countries and the selection of bacterial strains candidate as superior, highly specific inoculants for *Lotus* pastures in environmentally constrained soils in the region.

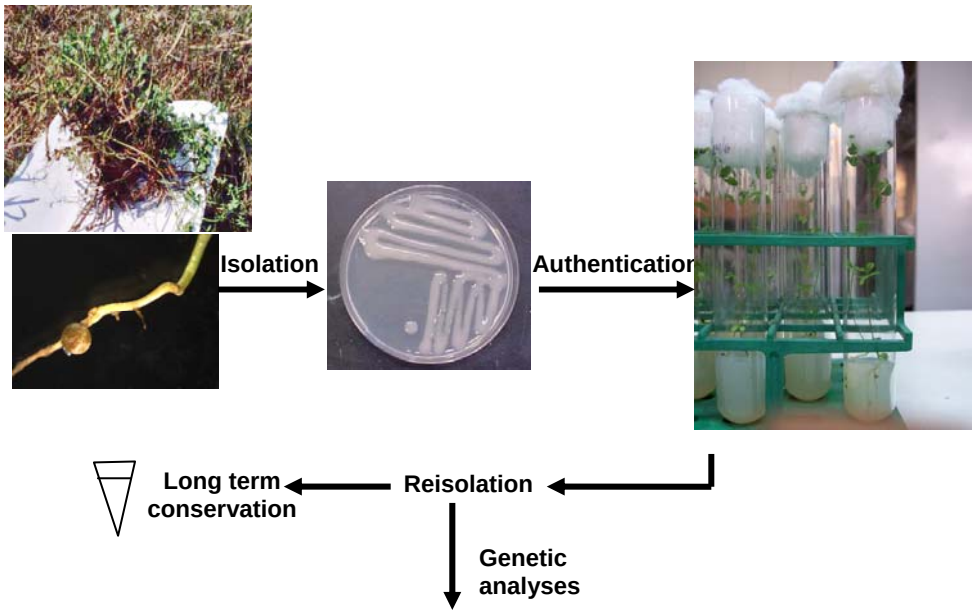

Figure 33. General methodology for isolation, analysis and conservation of *Lotus* rhizobia.

The Lotassa collection (http://microorganism.lotassa-databases.org/lotassamo/indexmo.html) contains more than 1,000 *Lotus* rhizobial strains isolated from constrained (drought, acidic, saline) soils of Argentina, Brazil, Chile and Uruguay, and from several European countries. Many of these isolates have been characterized at the phenotypic and genetic level, with particular emphasis in their symbiotic characteristics and tolerance to abiotic stresses (Figures 33 and 34).

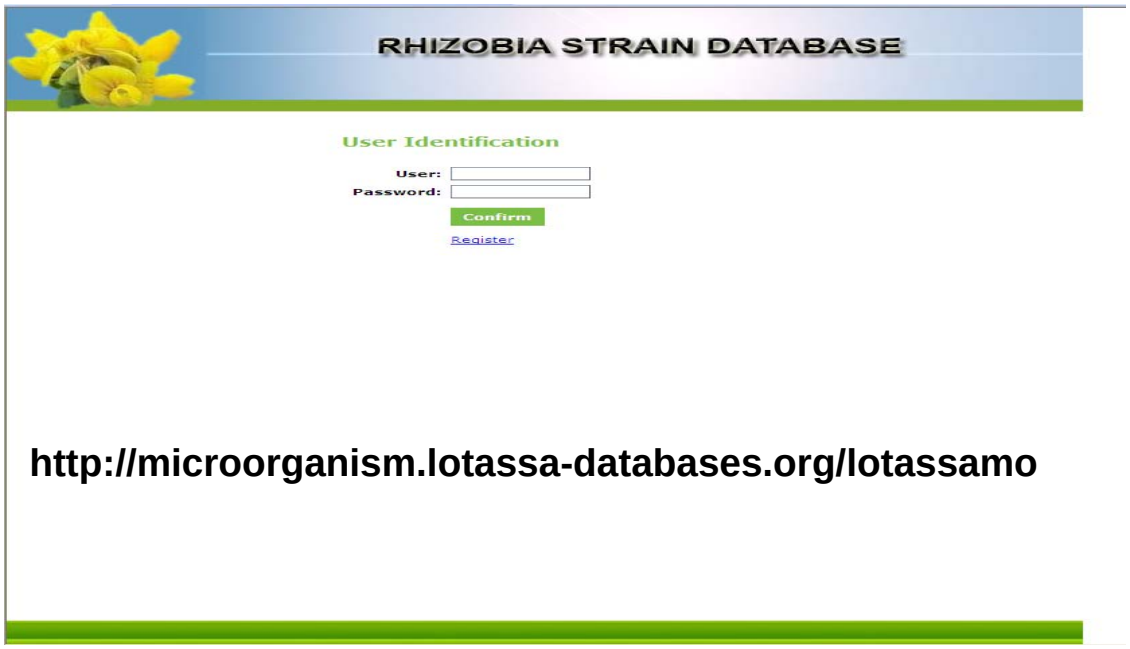

Figure 34. Access page to *Lotus*-rhizobia database.

6.2. Uruguay

L. corniculatus and *L. uliginosus* are traditionally used in Uruguay for grassland improvement, inoculated with rhizobia brought from Australia or New Zealand in the 60's, which has resulted in excellent economic and environmental profits. However, these inoculants were evaluated only in certain environments, without considering certain limitations like water deficits or the presence of native rhizobia, which are very competitive for nodulation but often poorly efficient for nitrogen fixation.

Thirteen sites across the country, chosen upon contrasting rain water availability and/or previous history of *Lotus* pastures and history of inoculation, were sampled to isolate near 300 rhizobial strains from nodules of *L. corniculatus* or *L. uliginosus*. Genetic diversity was above 50% according to ERIC-PCR genomic profiling (Figure 35). Taxonomic assignment of the rhizobia was based primarily on 16S rRNA gene sequencing, showing that *L. corniculatus* is nodulated mainly by bacteria of the genus *Mesorhizobium*, belonging to several species: *M. loti*, *M. amorphae*, *M. tianshanens*e, and *M. septentrionale*, whereas *L. uliginosus* is nodulated by bacteria of the genus *Bradyrhizobium*. As a general rule, rhizobia from one *Lotus* species were inefficient or even parasitic in the other *Lotus*. From symbiotic assays in greenhouse, several isolates were found as productive or even more efficient that currently recommended inoculants. Some of these isolates are currently under field evaluation.

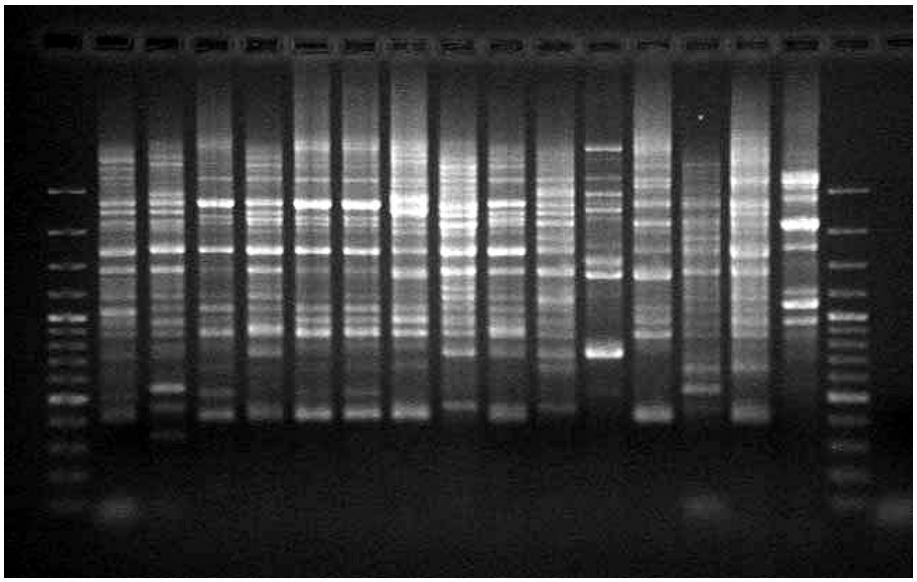

Figure 35. PCR-based genetic analyses of rhizobial strains.

6.3. Brazil

About 600 nodule isolates were collected from some 40 different locations throughout the state of Rio Grande do Sul, including acidic and non-acidic soils. In many sites there were no rhizobia nodulating *Lotus* or they were inefficient for nitrogen fixation. *Lotus* rhizobia were also isolated from native soils were *Lotus* had never been grown before, what can be explained because some of them were able to nodulate *Desmodium incanum*, a leguminous plant native from South America. Genetic diversity was high and preliminary results indicated that the Brazilian *Lotus* rhizobia would belong to at least two different genera, *Mesorhizobioum* and *Bradyrhizobium*. Symbiotic proficiency of isolates was tested with four different *Lotus* species: *Lotus corniculatus*, *Lotus tenuis*, *Lotus uliginosus* and *Lotus subbiflorus*. Many of the new isolates were found to be much more efficient that recommended strains, and plants reached higher productivity than with N fertilizer. Several of these proficient strains were also tolerant to acidity and high levels of aluminum, suggesting these could be good candidates as *Lotus* inoculants in acid soils. Some of these rhizobia were evaluated under field conditions.

6.4. Argentina

More than 100 bacterial isolates, from 3 different soil types in the Salado River Basin were obtained with *L. tenuis* as trap plant. Genetic diversity of these rhizobia was very high, and taxonomic studies indicated that *Lotus* rhizobia in this area belong to at least four different genera: *Mesorhizobium* (*M. ciceri, M. loti, M. tianshanense, M. amorphae, M. mediterraneum*) *Rhizobium* (*R. etli, R. gallicum, R. tropici) Agrobacterium (A. tumefaciens*) and *Aminobacter* (*A. aminovorans*). Regardless the species assignment, all these rhizobia showed a narrow host range, able to nodulate only *L. tenuis* but not *L. uliginosus*. Consistent with this, all of them seem to carry very related nodulation and nitrogen fixation genes. Under laboratory conditions, 19 isolates were found more symbiotically efficient that reference strains. Further, many of the strains were highly tolerant to salinity and alkalinity. A total of 18 isolates were found

potentially interesting as superior inoculants for *L. tenuis* in soils of the Salado River Basin, which however requires previous testing under field conditions.

6.5. Chile

158 nodule isolates were obtained from 15 locations (from VI to XIV region) in Chile, using *L. tenuis*, *L. corniculatus* or *L. uliginosus* as trap plants. Soil pHs ranged from 4.6 to 7.6. Genetic diversity was high according to ERIC-PCR profiling. Initial taxonomic assignment indicates these bacteria belong to genera *Mesorhizobium* and *Bradyrhizobium*. Some 70 unique isolates were tested for symbiotic performance and tolerance to acid pH. Several strains were found to perform better than N-fertilizer on their respective *Lotus* hosts, few of them were also acid-tolerant in laboratory media. Field tests with these strains are pending.

6.6. Spain and Portugal

Soil samples and nodules were collected from several locations in the Iberian Peninsula (Spain and Portugal) and the Canary Islands, resulting in a total of 272 isolates from different constrained soils and *Lotus* spp. Molecular identification of the isolates showed that *Lotus* spp. are nodulated by bacteria related to at least 4 different genera: *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Aminobacter*. The bradyrhizobia were found in Portuguese soils affected by heavy metal pollution and isolated from *L. uliginosus* and *L. corniculatus* nodules, were efficient in *L. uliginosus* but inefficient or partially efficient with *L. corniculatus*, and were related to two different species, *B. japonicum* and *Bradyrhizobium* genospecies α. These bacteria were also able to nodulate other legumes like *Lupinus*. The mesorhizobia were recovered from various *Lotus* spp. from soils of the Iberian peninsula affected by drought and salt, and from coastal soils of Canary islands, and could be ascribed to diverse *Mesorhizobium* species: *Mesorhizobium tianshanense/tarimense*, *M. chacoense/albiziae*, *M. plurifarium*, *M. ciceri*, and *M. alaghi*. Also Sinorhizobia (*Sinorhizobium meliloti*, *S. saheli/S. kostiense* and *S. fredii*) were symbionts of *L. lancerottensis, L. sessilifolius* or *L. kunkelii* in the Canary islands, some of these are considered endangered species. Some isolates, both from the peninsula and the Canary islands, were related to genus *Aminobacter* or showed an intermediate phylogenetic position between genera *Mesorhizobium* and *Aminobacter*. Phenotypic characterization indicated that all bradyrhizobia were salt-sensitive and well adapted to pH 6. The sinorhizobia were highly salt-resistant and pH tolerant, but showed a poor symbiotic performance on both *L. corniculatus* and *L. tenuis*. Approx. 10% of the mesorhizobia were high salt tolerant, efficient in *L. corniculatus* and adapted to pH ranging 6 to 8; a significant portion of mesorhizobial isolates were considered salt-dependent bacteria, showing better growth and symbiotic performance in presence of significantly high salt concentrations.

6.7. Europa Central y del Norte

The rhizobial symbionts of *L. corniculatus* and *L. uliginosus* natural populations in 21 locations of Germany, Sweden, Norway, Austria and Italy were analysed. 40 rhizobial isolates were studied and their host-ranges determined. *L. uliginosus* were usually nodulated by either *Bradyrhizobium* or *Rhodopseudomonas* bacteria, whereas *L. corniculatus* were strictly nodulated by *Rhizobium* or *Mesorhizobium*.

6.8. Conclusions

The results of Lotassa probably represent the world's most extensive and comprehensive study of *Lotus* symbionts. With an intercontinental scale, Lotassa was able to uncover the huge and greatly unexplored diversity of *Lotus* rhizobia in many countries and diverse environmental and agricultural situations. Researchers have been surprised by the huge biodiversity of bacteria able to establish symbiosis with *Lotus* spp., compared to what was known earlier (Figure 36). We learned that *Lotus* can be nodulated by genetically diverse bacteria belonging to not less than 6 different bacterial genera and tens of species. Biodiversity is one of the mainstays of biotechnology, and in this case, exploring natural diversity led to identify many bacterial strains with potentially high nitrogen-fixing efficiency with forage *Lotus* species in constrained environments (acid, drought, saline soils), thus providing new biologicals (bacteria) for future biotechnological exploitation.

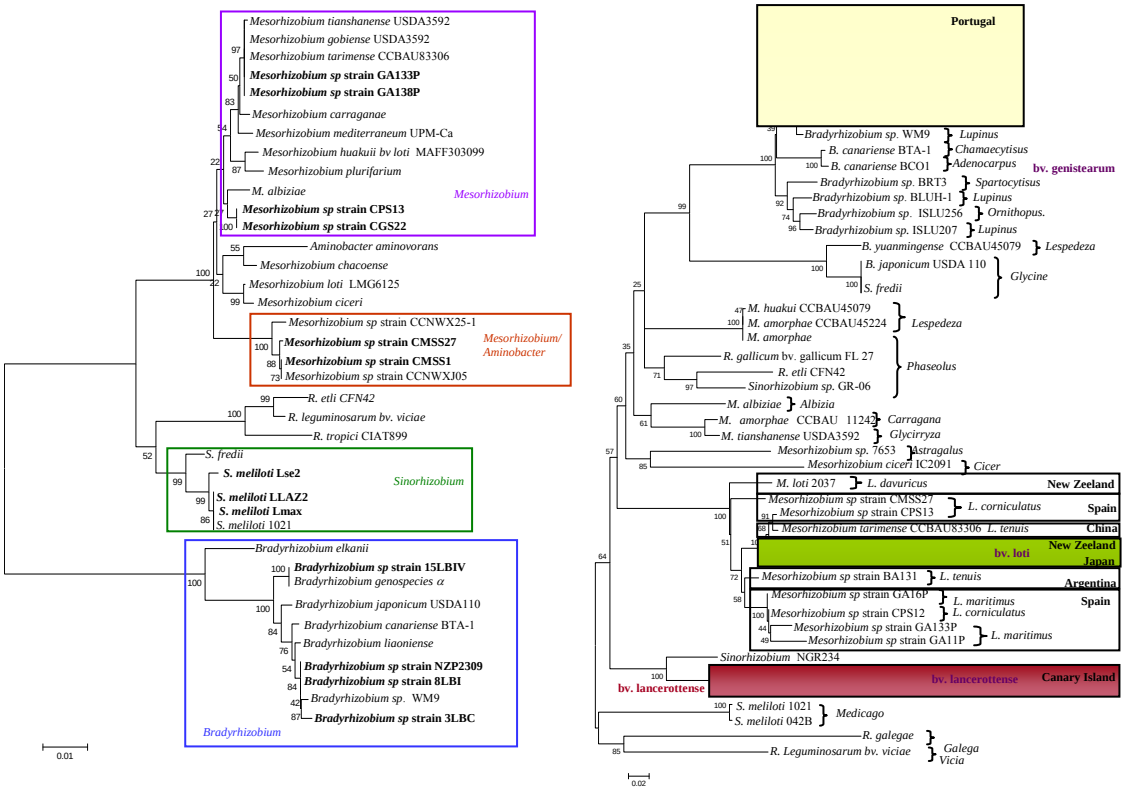

Figure 36. Phylogenetic analyses of *Lotus* rhizobia.

Chapter 7. Publications

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